Muscarinic m1 receptor-stimulated adenylate cyclase activity in Chinese hamster ovary cells is mediated by $G_s\alpha$ and is not a consequence of phosphoinositidase C activation

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The mechanism underlying muscarinic m1 receptor-mediated increases in adenosine 3',5'-cyclic monophosphate (cAMP) was investigated in Chinese hamster ovary (CHO) cells expressing human recombinant m1 muscarinic receptors (CHO-m1 cells). Stimulation of CHO-m1 cells with carbachol resulted in marked accumulation of $\text{Ins}(1,4,5)P_3$ and cAMP, in an atropine-sensitive manner, with EC_{50} values (log M) of -5.16 ± 0.06 and -3.93 ± 0.07 respectively. Basal and agonist-stimulated cAMP accumulation were unaffected by a 5 min pretreatment with $1 \mu\text{M}$ phorbol 12,13-dibutyrate and were not attenuated by pertussis toxin (100 ng/ml, 20 h). Agonist-stimulated cAMP accumulation was also observed in CHO-m1 cell membranes incubated in a buffer containing 100 nM free Ca^{2+} . Guanosine 5'-

$[\gamma\text{-thio}]\text{triphosphate}$ ($10 \mu\text{M}$) potentiated agonist-stimulated cAMP accumulation in CHO-m1 cell membranes, implicating a G-protein involvement in this response. Co-incubation of carbachol with forskolin ($10 \mu\text{M}$) produced a greater than additive accumulation of cAMP in CHO-m1 cells. Furthermore, a C-terminal-directed anti- $G_s\alpha$ serum attenuated both carbachol-stimulated (in CHO-m1 cell membranes) and isoprenaline-stimulated (in CHO- β_2 cell membranes) cAMP accumulation with a similar dose-dependency. These results suggest that muscarinic agonist-stimulated cAMP accumulation in CHO-m1 cells occurs via activation of $G_s\alpha$ and not as a consequence of phosphoinositidase C activation.

INTRODUCTION

Of the five muscarinic acetylcholine receptor genes identified to date [1–6], muscarinic m1, m3 and m5 receptors are thought to couple predominantly to the activation of phosphoinositidase C via the $G_{q/11}$ family of G-proteins [7,8]. However, m1 and m3 muscarinic receptor-mediated stimulation of adenylate cyclase activity has also been observed in some cell lines [9–12].

The mechanism of stimulation of adenylate cyclase activity appears to depend on the cell line in which the muscarinic receptors are expressed. Agonist-stimulation of adenylate cyclase activity via m1 muscarinic receptors in A9L cells appears to be a consequence of elevated Ca^{2+} levels following phosphoinositidase C activation [13]. In membrane preparations of rat olfactory bulb, muscarinic receptor-mediated stimulation of adenylate cyclase activity appears to be independent of free Ca^{2+} concentration and the response is inhibited after pertussis toxin (PTX) treatment [14], suggesting the involvement of G-protein $\beta\gamma$ subunits derived from PTX-sensitive G-proteins [15–17]. However, in contrast, muscarinic m3 receptor-mediated stimulation of adenylate cyclase activity in Chinese hamster ovary (CHO) cells appears to be insensitive to PTX pretreatment [10]. In our recent studies [18] we reported that agonist-mediated stimulation of m1 and m3 muscarinic receptors expressed in CHO cells leads to an accumulation of adenosine 3',5'-cyclic monophosphate (cAMP) in a PTX-insensitive manner but with EC_{50} values for carbachol that were much greater than those associated with $\text{Ins}(1,4,5)P_3$ accumulation and Ca^{2+} mobilization.

In the present study, muscarinic receptor-mediated stimulation of cAMP accumulation was measured in intact cell and mem-

brane preparations of CHO cells expressing recombinant m1 muscarinic receptors (CHO-m1 cells). The mechanism of carbachol-stimulated cAMP accumulation was investigated to determine whether this response is a consequence of phosphoinositidase C activation, or operates via a direct coupling of m1 muscarinic receptors with G_s . The results strongly suggest that m1 muscarinic receptors can couple with G_s to stimulate adenylate cyclase activity in CHO cells.

MATERIALS AND METHODS

Materials

ATP, atropine sulphate, *Bordetella* PTX, carbamoylcholine chloride (carbachol), ethylene glycol bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), forskolin, guanosine 5'- $[\gamma\text{-thio}]\text{triphosphate}$ (GTP[S]), Hepes, isoprenaline, isobutylmethylxanthine (IBMX), phorbol 12,13-dibutyrate (PDBU), 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine were purchased from Sigma Chemical Company. Growth media and supplements were obtained from GIBCO. $[\text{H}]\text{Ins}(1,4,5)P_3$ and $[\text{H}]N\text{-methylscopolamine}$ chloride were purchased from Amersham International. GTP (dilithium salt) was obtained from Boehringer. cAMP, $[\text{H}]\text{cAMP}$ and RM/1 anti- $G_s\alpha$ G-protein rabbit serum were purchased from NEN-DuPont. Anti- $G_{q/11}$ rabbit serum was a gift from Professor G. Milligan, Department of Biochemistry, Glasgow, U.K. $\text{Ins}(1,4,5)P_3$ was obtained from University of Rhode Island Foundation Chemistry Group, U.S.A. Ro 31-8220 was a gift from Roche Products Ltd., U.K. All other reagents were from Fisons Scientific Equipment.

Abbreviations used: cAMP, adenosine 3',5'-cyclic monophosphate; CHO, Chinese hamster ovary; CHO-m1 cells, CHO cells expressing human recombinant m1 muscarinic receptors; PTX, pertussis toxin; EGTA, ethylene glycol bis(β -aminoethyl ether) *N,N,N',N'*-tetra-acetic acid; GTP[S], guanosine 5'- $[\gamma\text{-thio}]\text{triphosphate}$; IBMX, isobutylmethylxanthine; PDBU, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate.

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Preparation of plasmid DNA and transfection procedures

Chinese hamster ovary (CHO-K1) cells transfected with cDNA encoding human m1 (CHO-m1) or m3 (CHO-m3) muscarinic receptors were obtained from Dr. N. J. Buckley, National Institute for Medical Research, Mill Hill, London. The methodology for preparation of plasmid DNA and transfection procedures were described previously [19]. CHO-m1 cells expressed m1 muscarinic receptors at a level of 1144 ± 192 fmol of [^{3} H]*N*-methylscopolamine binding sites/mg of protein ($n = 3$). The coding sequence for the human β_2 adrenoceptor was inserted into the vector pCEP-4 (Invitrogen) which was subsequently transfected into CHO-m3 cells using the calcium phosphate protocol [20]. Clones were isolated, grown in a selection medium containing 300 μ g/ml G418 and 600 units/ml hygromycin. In this manner a clone (CHO- β_2) was selected which produced a large isoprenaline-stimulated cAMP accumulation response, to be used as a control response for the action of anti- $G_{s\alpha}$ serum.

Cell culture

CHO cell clones were grown to confluence in α minimum essential medium (α MEM) supplemented with 10% (v/v) newborn calf serum, 100 i.u./ml penicillin, 100 μ g/ml streptomycin and 2.5 μ g/ml fungizone. Cells were incubated in a 5% CO₂ humidified incubator at 37 °C.

Radioligand binding

Carbachol displacement of [^{3} H]*N*-methylscopolamine binding (0.4 nM) was performed in membrane preparations of the CHO-m1 cells. CHO-m1 cells were grown to confluence and harvested using 10 mM Hepes, 0.02% EDTA, 0.9% NaCl, pH 7.4. Membranes were prepared by homogenization in 10 mM Hepes, 10 mM EDTA, pH 7.4, using a polytron tissue disrupter. The 40000 g membrane pellets were resuspended in binding buffer consisting of 10 mM Hepes, 1 mM MgCl₂ and 100 mM NaCl, pH 7.4. Experiments were incubated in the presence of 100 μ M GTP, for 1 h at 37 °C with a range of carbachol concentrations (1×10^{-7} – 1×10^{-2} M). Reactions were terminated by rapid vacuum filtration using Whatman GF/B filters. Filters were removed to scintillation vials and radioactivity was detected by liquid scintillation spectrometry.

Agonist-stimulated cAMP and Ins(1,4,5)*P*₃ accumulation experiments

CHO cell clones were grown to confluence and harvested using 10 mM Hepes, 0.02% EDTA, 0.9% NaCl, pH 7.4. Cells were washed twice and finally resuspended in Krebs-Hepes buffer with pH adjusted to 7.4 using 1 M NaOH. PTX pretreatment of cells consisted of growing cells for 20 h in medium containing 100 ng/ml PTX, prior to cell harvesting. cAMP accumulation experiments in CHO-m1 cell membranes (prepared as above) were performed in a cytosol-like buffer (CLB) consisting of 120 mM KCl, 2 mM Na₂ATP, 2.4 mM MgCl₂·6H₂O, 2 mM KH₂PO₄, 5 mM sodium succinate and 20 mM Hepes, pH 7.2, with the free [Ca²⁺] buffered to approx. 100 nM with 1–3 μ M EGTA.

Intact cell or membrane suspensions were then incubated in the presence or absence of a range of concentrations of carbachol, for defined time periods, in a final volume of 100 μ l, at 37 °C. Proteins were determined by the method of Lowry et al. [21], and each reaction tube contained approx. 30 μ g of protein. Reactions were terminated by the addition of 100 μ l of trichloroacetic acid (1 M).

Sample preparation and assay

Samples generated from experiments were centrifuged at 16000 g for 4 min. A sample (160 μ l) of supernatant was taken and added to 40 μ l of EDTA (10 mM stock) and 200 μ l of a mixture of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine (1:1, v/v). Samples were vortex-mixed and left for 15 min before being centrifuged at 16000 g for 4 min. An aliquot (100 μ l) of the upper phase was removed from each sample and added to tubes containing 50 μ l of NaHCO₃ (25 mM stock), correcting the pH of the sample to 7.8. cAMP-binding assays were performed using a radioreceptor assay with bovine adrenal cortical membranes [22]. Ins(1,4,5)*P*₃-binding assays were performed using a radioreceptor assay with bovine adrenal cortical membranes [23].

Anti- $G_{s\alpha}$ serum experiments

Membrane preparations of CHO-m1 or CHO- β_2 cells (30 μ g of protein/tube) were incubated in 30 mM Tris, 2.4 mM MgCl₂, 2 mM ATP and 1–3 μ M EGTA with either anti- $G_{s\alpha}$ serum or an equal protein concentration of pre-immune serum, for 2 h at 4 °C, prior to cAMP mass assays, in order to determine whether agonist-stimulated cAMP accumulation in these cell clones could be inhibited by anti- $G_{s\alpha}$ serum. Final serum protein concentrations used were 18 and 54 μ g/ml, representing a 300- and 100-fold dilution of the stock antiserum respectively.

Data analysis

K_{50} , EC_{50} , maximum and minimum values for dose–response curves were derived by computer-assisted curve-fitting (GraphPAD INPLOT Software Inc., San Diego, CA, U.S.A.). Results are expressed as the mean $\pm$ S.E.M. of the number of experiments indicated, each performed in duplicate. Attenuation of agonist-stimulated cAMP accumulation by anti- $G_{s\alpha}$ serum is expressed as the percentage attenuation compared with an identical protein concentration of pre-immune serum. Statistical evaluations were performed using Student's *t*-tests.

RESULTS

Agonist-stimulated cAMP accumulation in intact CHO-m1 cells

Maximal carbachol (1 mM)-stimulated cAMP accumulation, in CHO-m1 cells, occurred after 5 min incubation at 37 °C, reaching a peak accumulation of 370 ± 90 pmol/mg of protein from a basal level of 11 ± 1 pmol/mg of protein ($n = 3$). Basal and carbachol-stimulated cAMP accumulation were potentiated in CHO-m1 cells after 30 min pretreatment with 1 mM IBMX. Basal cAMP levels, after 5 min incubation, were increased from 9 to 35 pmol/mg of protein. Carbachol-stimulated cAMP levels were increased from 570 to 888 pmol/mg of protein (mean of two experiments). All further studies were carried out in the absence of IBMX. Carbachol dose–response curves were performed in CHO-m1 cells, after 5 min incubation at 37 °C, both in the presence or absence of PTX pretreatment (100 ng/ml for 20 h) as reported previously [18]. Maximal carbachol-stimulated cAMP accumulation in the absence of PTX pretreatment was 306 ± 2 pmol/mg of protein from a basal level of 13 ± 2 pmol/mg of protein, a 24-fold stimulation ($n = 3$). After PTX pretreatment, carbachol-stimulated cAMP accumulation was significantly enhanced compared with control data ($P < 0.05$), achieving a maximal accumulation of 447 ± 67 pmol/mg of protein from a basal level of 16 ± 5 pmol/mg of protein ($n = 3$). The EC_{50} (log M) values for this carbachol-stimulated cAMP accumulation response were not significantly different between control and

Table 1 Effect of a 5 min preincubation with PDBU (1 μ M) on basal and carbachol (1 mM)-stimulated cAMP accumulation (pmol/mg of protein) in CHO-m1 cells, after 5 min at 37 °C

Data are expressed as the mean $\pm$ S.E.M. of four experiments, each performed in duplicate.

	cAMP accumulation (pmol/mg of protein)	
	– PDBU	+ PDBU
Basal	11 $\pm$ 4	13 $\pm$ 4
Carbachol	384 $\pm$ 77	337 $\pm$ 85

PTX-pretreated cells, being -3.93 ± 0.07 and -3.94 ± 0.03 respectively ($n = 3$).

Pretreatment of CHO-m1 cells with 1 μ M PDBU for 5 min, prior to 1 mM carbachol stimulation, had no significant effect on carbachol-stimulated cAMP accumulation over 20 min, suggesting that activation of protein kinase C played no role in the activation of adenylate cyclase in these cells (Table 1).

Co-incubation of carbachol (1 mM) with 10 μ M forskolin for 5 min produced a greater than additive cAMP accumulation response ($P < 0.05$, by unpaired Student's *t*-test) in CHO-m1 cells (Table 2).

Agonist-stimulated Ins(1,4,5) P_3 accumulation in CHO-m1 cells

Carbachol-stimulated Ins(1,4,5) P_3 accumulation dose–response curves were performed in CHO-m1 cells after 10 s incubation corresponding to the peak phase of Ins(1,4,5) P_3 accumulation [18,24]. Maximal carbachol-stimulated Ins(1,4,5) P_3 accumulation was 384 ± 40 pmol/mg of protein from basal levels of 47 ± 12 pmol/mg of protein ($n = 4$). The EC_{50} (log M) for this carbachol-mediated response was -5.16 ± 0.06 (Figure 1) which was similar to the EC_{50} for the plateau phase of Ins(1,4,5) P_3 accumulation after 10 min incubation with carbachol [18].

Radioligand binding in CHO-m1 cell membranes

Carbachol displacement of 0.4 nM [^{3} H]*N*-methylscopolamine binding to CHO-m1 cell membranes, in the presence of a maximally effective concentration of GTP (100 μ M), produced a binding curve which was best fitted to a single low-affinity carbachol-binding site [K_{50} (log M) of -3.69 ± 0.03 ($n = 4$)]. The occupancy–response relationships between carbachol binding, carbachol-stimulated Ins(1,4,5) P_3 accumulation and carbachol-stimulated cAMP accumulation are shown in Figure 1. The

Table 2 Carbachol (1 mM)- and forskolin (10 μ M)-stimulated cAMP accumulation (pmol/mg of protein) in intact CHO-m1 cells after 5 min incubation at 37 °C

Data are expressed as the mean $\pm$ S.E.M. of three experiments each performed in duplicate. The carbachol + forskolin response was significantly greater than additive compared with the sum of the two individual responses (* signifies $P < 0.05$).

	cAMP accumulation (pmol/mg of protein)
Basal	4 $\pm$ 2
Carbachol	440 $\pm$ 42
Forskolin	357 $\pm$ 45
Carbachol + forskolin	1083 $\pm$ 44*

Figure 1 Occupancy–response relationship for carbachol-stimulated Ins(1,4,5) P_3 accumulation (■) and cAMP accumulation (◆) in CHO-m1 cells and carbachol displacement of [^{3} H]*N*-methylscopolamine binding (●) in CHO-m1 cell membranes in the presence of 100 μ M GTP

Data are expressed as the means of the maximal percentage response for the number of experiments indicated.

Figure 2 Time course for basal (○, □) and (1 mM) carbachol-stimulated cAMP (●, ■) accumulation in CHO-m1 membranes, at 37 °C, in the presence (□, ■) and absence (○, ●) of 10 μ M GTP[S]

Incubations were performed in a cytosol-like buffer containing 2 mM ATP with free Ca^{2+} buffered to approx. 100 nM with 1–3 μ M EGTA. Data are expressed as the means $\pm$ S.E.M. of three experiments each performed in duplicate.

carbachol-stimulated Ins(1,4,5) P_3 accumulation response lies approx. 30-fold to the left of the carbachol occupancy curve, whereas the carbachol-stimulated cAMP accumulation response almost overlies the apparent carbachol occupancy curve. Thus, for example, 30 μ M carbachol produces a maximal stimulation of Ins(1,4,5) P_3 accumulation while producing less than 20 % of the possible maximal cAMP accumulation response.

Agonist-stimulated cAMP accumulation in CHO-m1 cell membranes

To determine whether carbachol-stimulated cAMP accumulation was a consequence of elevated free cytosolic Ca^{2+} levels, carbachol-stimulated cAMP accumulation was performed in CHO-m1 cell membranes, in a cytosol-like buffer containing

Figure 3 Carbachol-stimulated cAMP accumulation in the presence and absence of $10 \mu\text{M}$ GTP[S], in CHO-m1 cell membranes after 20 min incubation at 37°C

Data are expressed as the means $\pm$ S.E.M. of three experiments each performed in duplicate.

2 mM ATP and with free Ca^{2+} levels buffered to approx. 100 nM with 1–3 μM EGTA. In this way free cytosolic Ca^{2+} levels could be buffered to the approximate resting levels of cytosolic Ca^{2+} found within CHO cells [25]. Carbachol (1 mM) produced a 4-fold increase in cAMP accumulation above basal levels, after 20 min at 37°C (Figure 2). In the presence of $10 \mu\text{M}$ GTP[S], cAMP accumulation was elevated in a linear manner up to 20 min incubation. In the presence of $10 \mu\text{M}$ GTP[S], carbachol (1 mM) stimulated cAMP accumulation in a greater than additive manner, after 20 min, suggesting the involvement of a G-protein in this muscarinic receptor-mediated response. Carbachol dose-response curves constructed both in the presence and absence of $10 \mu\text{M}$ GTP[S] are shown in Figure 3. In the absence of GTP[S], carbachol (10 mM) produced a maximal stimulation of cAMP accumulation of 262 ± 18 from basal levels of 63 ± 11 pmol/mg of protein with an EC_{50} (log M) of -3.76 ± 0.19 . In the presence of GTP[S] ($10 \mu\text{M}$), carbachol (10 mM) produced a maximal stimulation of cAMP accumulation of 1870 ± 78 from basal levels of 1376 ± 65 pmol/mg of protein with an EC_{50} (log M) of -4.70 ± 0.17 . In both cases the carbachol-stimulated cAMP accumulation response was inhibited in the presence of $10 \mu\text{M}$ atropine. Carbachol-stimulated cAMP accumulation in CHO-m1 membranes was increased approx. 2.5-fold, in the presence of $10 \mu\text{M}$ GTP[S] compared with control data. The EC_{50} for carbachol, in the absence of GTP[S], was not significantly different from that found in intact CHO-m1 cells. However, in the presence of $10 \mu\text{M}$ GTP[S], the potency of carbachol for this response was increased by approx. 9-fold.

Under identical experimental conditions, no carbachol-stimulated $\text{Ins}(1,4,5)\text{P}_3$ mass accumulation could be detected in CHO-m1 membranes, suggesting that there was little if any phosphoinositidase C activity in membrane preparations of these cells.

Effect of anti- $\text{G}_s\alpha$ serum on agonist-stimulated cAMP accumulation

In order to ascertain any possible functional inhibition of anti- $\text{G}_s\alpha$ serum on muscarinic receptor-mediated cAMP accumu-

Table 3 Dose-dependent inhibition of agonist-stimulated cAMP accumulation with anti- $\text{G}_s\alpha$ serum, in CHO-m1 and CHO- β_2 cell membranes

Membranes were incubated for 2 h at 4°C with equal protein concentrations of either anti- $\text{G}_s\alpha$ serum or pre-immune serum, followed by a 20 min incubation at 37°C , with either 1 mM carbachol (in CHO-m1 membranes) or 10 nM isoprenaline (in CHO- β_2 membranes). Data are expressed as the mean $\pm$ S.E.M. of the percentage inhibition of the cAMP response compared with an equal dilution of the pre-immune serum. Abbreviation: nd, not determined.

Anti- $\text{G}_s\alpha$ serum	Inhibition of cAMP response (%)	
	CHO-m1	CHO- β_2
1:300 dilution (18 μg of protein/ml)	20 ± 2 ($n = 3$)	6 ± 3 ($n = 3$)
1:100 dilution (54 μg of protein/ml)	37 ± 5 ($n = 3$)	26 ± 2 ($n = 3$)
1:30 dilution (180 μg of protein/ml)	44 ($n = 2$)	nd

lation, it was first important to establish a positive control on a well known $\text{G}_s\alpha$ -mediated stimulation of adenylate cyclase activity. CHO cells were transfected with the cDNA for β_2 adrenoreceptors and the resulting clone (CHO- β_2) produced a large isoprenaline-stimulated cAMP accumulation response which was sensitive to β -adrenoreceptor antagonists (K. Ellis, unpublished work). Isoprenaline (10 nM) produced approximately the same level of cAMP accumulation in CHO- β_2 cell membranes as did 1 mM carbachol in CHO-m1 cell membranes, after 20 min (results not shown). For effective functional activity of the antiserum, the buffer was changed to a Tris- Mg^{2+} buffer with added ATP and free Ca^{2+} buffered to approx. 100 nM. This change in buffer had no significant effect on agonist-stimulated levels of cAMP accumulation after 20 min in either cell line (results not shown), but enhanced the inhibitory effect of the antiserum on the cAMP accumulation response.

Pretreatment of cell membranes with pre-immune serum resulted in a small but significant attenuation of agonist-mediated cAMP accumulation (by approx. 18% at a 1:300 dilution and 25% at a 1:100 dilution) both in CHO-m1 and CHO- β_2 cell membranes. However, the dose-dependent attenuation of the agonist-stimulated cAMP accumulation responses were markedly greater with the anti- $\text{G}_s\alpha$ serum than with the pre-immune serum (Table 3). Anti- $\text{G}_{q/11}$ sera produced no significant attenuation of the carbachol-stimulated cAMP response in CHO-m1 cell membranes compared with the pre-immune serum up to a 1:100 dilution of the respective antisera (results not shown).

DISCUSSION

Agonist-stimulated cell signalling in tissues is often complicated by the presence of multiple receptor subtypes for which the agonist has very little selectivity. Expression of recombinant receptors in cell lines which do not endogenously express these receptors, provides a model system for studying single subtypes of receptor in isolation. In this way, specific receptor-mediated cell signalling can be investigated as long as the cell line used has the necessary components to transduce the receptor signal into a cellular response. In the present study, CHO cells expressing recombinant m1 muscarinic receptors that efficiently couple to phosphoinositidase C were used to investigate the mechanism of agonist-mediated cAMP accumulation which has been previously reported [11,12,18].

IBMX potentiated basal and carbachol-stimulated cAMP accumulation in CHO-m1 cells, suggesting that the response

resulted from direct activation of adenylate cyclase rather than via phosphodiesterase inhibition. Stimulation of adenylate cyclase can result from activation of phosphoinositidase C by a number of mechanisms depending on the isoforms of adenylate cyclase present. Protein kinase C activation resulting from diacylglycerol accumulation can stimulate type-2 adenylate cyclase (and also types 1 and 3 to a lesser extent) [15]. $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} mobilization can elevate cytosolic Ca^{2+} levels, thus activating type-1 and -3 adenylate cyclase via Ca^{2+} /calmodulin [15]. Alternatively, receptor-mediated stimulation of adenylate cyclase could result from a direct coupling with $\text{G}_s\alpha$ or via G-protein $\beta\gamma$ subunits which can activate type-2 and -4 adenylate cyclase in the presence of activated $\text{G}_s\alpha$ [15–17].

In cell systems where muscarinic receptor-stimulated adenylate cyclase activity is mediated by Ca^{2+} -calmodulin activity, the agonist dose-response curves for Ca^{2+} elevation and cAMP accumulation are very similar [26]. However, in CHO cells expressing recombinant m1 or m3 muscarinic receptors, agonists are less potent at stimulating adenylate cyclase activity compared with stimulating phosphoinositidase C activity or elevating intracellular free Ca^{2+} levels [10–12,18]. At similar levels of receptor expression in CHO cells, m1 and m3 muscarinic receptor-stimulation mediates comparable activation of phosphoinositidase C, while m1 muscarinic receptor-stimulation produces a much greater accumulation of cAMP than m3 muscarinic receptors [11,18]. Jones et al. [10] also found that m3 or m5 muscarinic receptors, expressed in CHO cells, produced similar levels of total inositol phosphate accumulation, but cells expressing m3 muscarinic receptors (at a relatively high expression level) produced a much larger agonist-mediated cAMP response than m5 muscarinic receptors. Together, these results suggest that the muscarinic agonist-stimulated cAMP response, in CHO cells, may not be simply a consequence of phosphoinositidase C activation. This would be consistent with results obtained from the present studies. PDBU had no significant effect on either basal or carbachol-stimulated cAMP accumulation although a similar pretreatment of SH-SY5Y cells has been shown to attenuate agonist-stimulated $\text{Ins}(1,4,5)\text{P}_3$ accumulation, suggesting that this pretreatment was adequate to stimulate protein kinase C activation [24]. Therefore, protein kinase C activation appears to play no significant role in m1 muscarinic receptor-mediated cAMP accumulation and may indicate the apparent absence of type-2 adenylate cyclase in CHO cells. However, Gurwitz et al. [11] reported that a 1 h pretreatment with $0.1\text{ }\mu\text{M}$ phorbol 12-myristate 13-acetate (PMA) greatly reduced the muscarinic agonist-stimulated cAMP response in CHO-m1 cells. In that study, carbachol was 30-fold more potent at stimulating phosphoinositidase C activity compared with stimulating cAMP accumulation, suggesting that a near maximal activation of phosphoinositidase C could be stimulated (with $10\text{ }\mu\text{M}$ carbachol) without producing a stimulation of cAMP accumulation. Therefore, it is unlikely that the carbachol-stimulated cAMP response resulted as a consequence of protein kinase C activation. Stimulation of muscarinic m1 receptor-mediated cAMP accumulation in A9L cells was also shown to be inhibited by PMA [13]. However, in that study the potency difference for carbachol to stimulate phosphoinositidase C activity and adenylate cyclase activity was only 2–3-fold. The carbachol-stimulated cAMP response in membrane preparations of these cells was also insensitive to the addition of GTP, suggesting that in A9L cells carbachol-stimulated adenylate cyclase activity may not be directly coupled through a G-protein. In the present study, the protein kinase C inhibitor Ro 31-8220 produced an inhibition of carbachol-stimulated cAMP accumulation but was also found to displace [^3H]*N*-methylscopolamine

binding with a similar affinity, suggesting that the inhibitory effect of this compound was due to its (albeit weak) muscarinic antagonist activity (N. T. Burford, unpublished work).

To evaluate a possible role of elevated cytosolic Ca^{2+} in muscarinic m1-mediated cAMP accumulation, carbachol-stimulated cAMP accumulation was measured in membrane preparations where free Ca^{2+} levels were maintained at approx. 100 nM with $1\text{--}3\text{ }\mu\text{M}$ EGTA. Carbachol produced a 4-fold increase in cAMP accumulation above basal levels after 20 min incubation, suggesting that elevated cytosolic Ca^{2+} levels are not necessary to evoke this m1 muscarinic receptor-mediated response. These findings are in agreement with those of Gurwitz et al. [11] who found that EGTA and BAPTA-AM [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester] did not inhibit the carbachol-stimulated cAMP response in intact CHO-m1 cells. In the present study, the carbachol-stimulated cAMP response was enhanced by $10\text{ }\mu\text{M}$ GTP[S], implicating G-protein-mediated adenylate cyclase activity. Under identical conditions, no carbachol-stimulated $\text{Ins}(1,4,5)\text{P}_3$ accumulation could be detected, possibly indicating a lack of phosphoinositidase C activity in membrane preparations of these cells. Therefore, carbachol-stimulated cAMP accumulation was detected in the absence of phosphoinositidase C activity in CHO-m1 membranes, suggesting that the stimulation of adenylate cyclase was not a consequence of phosphoinositidase C activity.

Carbachol-stimulated cAMP accumulation in CHO-m1 cells was not attenuated (but was slightly enhanced) by PTX pretreatment, suggesting that the G_i family of G-proteins and their associated $\beta\gamma$ subunits were not involved in this mechanism. The small potentiation of carbachol-stimulated cAMP accumulation after PTX pretreatment may be a consequence of a tonic inhibitory effect of G_i on adenylate cyclase which is abolished after PTX pretreatment. Pretreatment with PTX has been shown to potentiate forskolin-stimulated cAMP accumulation in a number of cell lines even in the absence of an inhibitory hormone [27]. The PTX-sensitivity of this response was important to define considering the evidence that some agonist-stimulated [^{35}S]GTP[S] binding to G-proteins in CHO-m1 cell membranes can be inhibited by PTX pretreatment [28,29].

The potency of carbachol to stimulate cAMP accumulation was not significantly different between membrane preparations of CHO-m1 cells (in the absence of GTP[S]) and intact cells, suggesting that the mechanism of muscarinic receptor-mediated cAMP accumulation was identical under both experimental conditions. Interestingly, co-incubation of carbachol with $10\text{ }\mu\text{M}$ forskolin produced a greater than additive cAMP accumulation response in intact CHO-m1 cells. Such synergistic cAMP accumulation responses have been observed between forskolin and activated $\text{G}_s\alpha$ and between forskolin and agonists that stimulate receptors coupled to adenylate cyclase via G_s [30,31].

Several studies have shown that receptors coupling predominantly to one G-protein family can also couple with other G-proteins, though less efficiently. For example, α_2 adrenoreceptors coupling predominantly to G_i can also couple with G_s in CHO cells [32,33], producing a stimulation of adenylate cyclase activity at high agonist concentrations in a manner which was also dependent on higher receptor expression levels. Muscarinic m4 receptors in HEK-293 cells can also couple with both G_i and G_s G-proteins [34]. Similarly, neurotensin receptors [35] and α_{1B} -adrenergic receptors [36] in CHO cells, and thyroliberin receptors in GH $_3$ cells [37] couple predominantly with $\text{G}_{q/11}$ family G-proteins but can also couple with G_s . A possible m1 muscarinic receptor coupling to G_s in CHO cell membranes was therefore investigated by observing whether anti- $\text{G}_s\alpha$ serum could be used to attenuate carbachol-stimulated cAMP accumulation in CHO-

m1 cell membranes. The anti- G_{α} serum (RM/1) has previously been characterized in immunoblots and functional studies [33,35,38]. Furthermore, as a control, anti- G_{α} serum-mediated attenuation of isoprenaline-stimulated cAMP accumulation was also assessed in CHO- β_2 cell membranes. The anti- G_{α} serum was found to attenuate both carbachol and isoprenaline-stimulated cAMP accumulation in a dose-dependent manner in CHO-m1 and CHO- β_2 cell membranes respectively, suggesting an involvement of G_{α} in mediating these two agonist-mediated responses in CHO cells. An identical concentration of anti- $G_{\alpha/11}$ sera had no effect on carbachol-stimulated cAMP levels in CHO-m1 cell membranes above that of the pre-immune serum.

In summary, the present study provides strong evidence that m1 muscarinic receptors expressed in CHO cells can couple, not only with $G_{\alpha/11}$ mediating the stimulation of phosphoinositidase C activity, but also to G_s mediating the stimulation of adenylate cyclase activity. How this evidence relates to m1 muscarinic receptors expressed in other cell lines or tissues will probably depend upon the receptor-G-protein stoichiometry within the cell used and the isoforms of adenylate cyclase expressed. The fact that the carbachol-stimulated cAMP accumulation response appears to overlay the apparent affinity of carbachol for m1 muscarinic receptors in CHO-m1 cells suggests that there is little, if any, apparent receptor-reserve for this response, even at receptor expression levels that can be found physiologically (i.e. 1 pmol/mg of protein) [7]. Thus, differences in receptor expression would be expected to influence the maximal cAMP accumulation response to muscarinic agonists and agonist-stimulated cAMP accumulation may not be detectable below a threshold expression level of receptors. Therefore, manipulation of receptor and/or G-protein expression could result in different second-messenger responses between tissues, as proposed recently for the α_{1B} -adrenergic receptor [36]. However, the present data do suggest that m1 muscarinic receptors, coupling predominantly with $G_{\alpha/11}$ to activate the phospholipase C, protein kinase C and Ca^{2+} cascade, can also couple, albeit less efficiently, with G_s to stimulate cAMP accumulation. Muscarinic receptor-mediated cAMP accumulation via this, or indirect, mechanisms may contribute to the regulation of muscarinic receptor-mediated signalling via cAMP-dependent protein kinase [39,40].

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