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Agonist activation of transfected human M1 muscarinic acetylcholine receptors in CHO cells results in down-regulation of both the receptor and the α subunit of the G-protein G_q

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CHO cells stably transfected with cDNA encoding the human M1 muscarinic acetylcholine (HM1) receptor were treated with the cholinergic agonist carbachol at various concentrations for differing times. Levels of the HM1 receptor and of a range of G-proteins were subsequently measured. Carbachol treatment of the transfected cells caused a substantial down-regulation of cellular levels of the α subunit of G_q ($G_q\alpha$), but did not significantly alter cellular levels of the α subunits of G_s or G_i2. A small decrease in levels of G-protein β-subunit was also produced. Parallel assessment of agonist-induced down-regulation of the HM1 receptor demonstrated that it was lost in concert with the G-protein. Similar concentrations of carbachol (5 μM) were required to produce half-maximal stimulation of inositol phosphate generation and loss of each of the HM1 receptor and $G_q\alpha$, and half-maximal losses of both receptor and $G_q\alpha$ were produced by 3 h of treatment with 1 mM-carbachol. By contrast, treatment of the non-transfected parental CHO cells, which do not express detectable levels of the receptor, with carbachol had no effect on cellular $G_q\alpha$ levels. Concurrent treatment of the HM1-expressing CHO cells with carbachol and cycloheximide indicated that suppression of protein synthesis *de novo* did not mimic the effect of carbachol, and hence even complete inhibition of transcription of the $G_q\alpha$ gene and/or translation of pre-existing $G_q\alpha$ mRNA could not account for the agonist-induced effect. We have previously noted that cellular levels of both $G_s\alpha$ [McKenzie and Milligan (1990) J. Biol. Chem. **265**, 17084–17093] and the α subunits of the pertussis-toxin-sensitive G-proteins G_i1, G_i2 and G_i3 [Green, Johnson and Milligan (1990) J. Biol. Chem. **265**, 5206–5210] can be regulated in certain cell systems by agonist activation of receptors expected to interact with these G-proteins. These results demonstrate that the same is true of $G_q\alpha$ and suggest that agonist-induced co-ordinate loss of receptors and associated G-proteins may be a more common feature than has been appreciated to date.

INTRODUCTION

A considerable family of heterotrimeric G-proteins functions to allow communication between trans-plasma-membrane receptors and effector systems involved in the synthesis of intracellular secondary messengers [1]. Ca^{2+}-mobilizing receptors produce stimulation of a phosphoinositidase C by activation of a G-protein often referred to as G_p [2]. Stimulation of phosphoinositidase C results in the generation of the water-soluble secondary messenger inositol 1,4,5-trisphosphate, and it is this molecule which elicits the release of Ca^{2+} from intracellular stores [3]. As in most systems G_p is not modified by the ADP-ribosyltransferase activities of either pertussis or cholera toxins [2], then for some time it was difficult to identify this G-protein. However, the purification of 42 and 43 kDa polypeptides from liver and brain able to cause the activation of phospholipase Cβ1 indicated that these polypeptides were likely to represent the α subunit of G_p-like G-proteins [4,5]. Comparison of partial amino acid sequences from these proteins with the predicted amino acid sequences from a series of novel G-protein α-subunit cDNA species [6–8] indicated that these polypeptides are part of the G_q/G11 series of G-proteins.

Chronic agonist activation of cell-surface receptors usually results in a decrease in the density of the receptor [9]. This process is termed down-regulation. Complex regulation of the activities and levels of individual components of cellular signalling cascades might be expected to occur during such chronic exposure to receptor agonists [10], and we have noted previously that sustained activation of a prostanoid receptor on neuroblastoma × glioma hybrid NG108-15 cells, which interacts with G_s to cause activation of adenylate cyclase, leads to a marked decrease in cellular levels of the α subunit of G_s [11]. Furthermore, activation of receptors for some, but not all, anti-lipolytic agents on rat white fat-cells in tissue culture leads to a decrease in levels of each of the pertussis-toxin-sensitive G-proteins G_i1, G_i2 and G_i3 in a manner which could be reversed by the removal of the anti-lipolytic stimulus [12,13].

Cross-regulation of cellular G-protein levels have also been recorded. In S49 lymphoma cells, elevation of intracellular cyclic AMP results in an up-regulation of levels of the α subunit of G_i2 [14], the G-protein responsible for transduction of receptor-mediated inhibition of adenylate cyclase [15,16].

In the present paper we study the effect of sustained exposure of CHO cells expressing the human M1 muscarinic acetylcholine receptor [17] to the cholinergic agonist carbachol on cellular levels of G-proteins and of the receptor. We record that such exposure results in marked down-regulation of G_q, but has little effect on membrane associated levels of other G-proteins.

EXPERIMENTAL

Materials

[^{3}H]Quinuclidinyl benzilate (QNB), [α-^{32}P]ATP, cyclic [^{3}H]AMP and *myo*-[^{3}H]inositol were from Amersham International.

Abbreviation used: QNB, quinuclidinyl benzilate.

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Table 1 Generation and specificities of anti-G-protein antisera

The antisera were generated in New Zealand White rabbits by using a conjugate of the synthetic peptide and keyhole-limpet haemocyanin as detailed under 'Methods'. $G_i1\alpha$ and $G_i2\alpha$ have identical C-terminal decapeptides, and thus antiserum SG1 identifies each of these polypeptides equally. Transducin α is also identified by antiserum SG1, but, as this G-protein is restricted in distribution to photoreceptor-containing tissues, the antiserum can be used as a probe of G_i1 or G_i2 in all other locations. We did not record any cross-reactivity on immunoblots with *Escherichia coli*-expressed G-protein α subunits of antiserum SG1 with $G_i3\alpha$ or of antiserum I3B with either $G_i1\alpha$ or $G_i2\alpha$ (see [13,45] for details). The specificity of antiserum CQ2 for G_q/G11 has previously been established [28,39].

Antiserum	Peptide employed	Corresponding G-protein sequence	Antiserum identifies
CQ2	QLNLKEYNLV	Gqα 351–360, G11α 350–359	Gq, G11
IQ1	EKVSAFENPYVDAIKS	$G_q\alpha$ 119–134	Gq
SG1	KENLKDCGLF	Transducin α 341–350	Transducin, G_t1, G_i2
I3B	KNNLKECGLY	$G_i3\alpha$ 345–354	G_i3
CS1	RMHLRQYELL	$G_s\alpha$ 385–394	G_s
BN3	MSELDQLRQE	β1 1–10	β

Carbachol and atropine were from Sigma. All materials for tissue culture were from GIBCO/BRL.

Methods

Generation of the HM1-expressing CHO cell line

A 1.79 kb *Bam*H1-*Bst*E2 fragment of the human M1 gene containing the complete coding sequence and 127 bases of 3′ untranslated sequence was ligated with 0.96 kb of 3′ untranslated rat M1 sequence in the pCD vector [18,19]. CHO-K1 cells were co-transfected with pCDneo by calcium phosphate precipitation [20], and resistant colonies were selected in the presence of G418 (400 μg/ml). Single colonies were isolated by using cloning rings and propagated to generate continuous cell lines. Positive colonies were identified by binding of [^{3}H]scopolamine methyl chloride [21].

Cell culture

Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn-calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in 5% CO_2 at 37 °C. Cells were grown in 75 cm^2 tissue-culture flasks and were harvested just before confluency. For these experiments, cells between passages 5 and 15 were used. In many cases, cells were treated for different times or with different concentrations of the cholinergic agonist carbachol.

Membranes were prepared from the cells by homogenization with a Teflon-on-glass homogenizer and differential centrifugation as described for a variety of other cells [22]. Frozen cell pellets were suspended in 5 ml of 10 mM Tris/HCl/0.1 mM EDTA, pH 7.5 (buffer A), and rupture of the cells was achieved with 25 strokes of a hand-held Teflon-on-glass homogenizer. The resulting homogenate was centrifuged at 500 ***g*** for 10 min in a Beckman L5-50B centrifuge with a Ti 50 rotor, to remove unbroken cells and nuclei. The supernatant fraction from this treatment was then centrifuged at 48000 ***g*** for 10 min, and the pellet from this treatment washed and resuspended in 10 ml of buffer A. After a second centrifugation at 48000 ***g*** for 10 min, the membrane pellet was resuspended in buffer A to a final protein concentration of 1–3 mg/ml and stored at −80 °C until required.

Binding experiments

These were performed at 30 °C for 30 min in 10 mM Tris/HCl/50 mM sucrose/20 mM $MgCl_2$, pH 7.5 (buffer B). In saturation experiments using [^{3}H]QNB, the concentration of ligand was varied between 50 pM and 1.0 nM. Non-specific binding was defined in all cases by parallel assays containing 10 μM atropine. Non-specific binding increased with [^{3}H]ligand concentration in a linear manner. Binding experiments were terminated by rapid filtration through Whatman GF/C filters, followed by three washes of the filter with ice-cold buffer B (5 ml each). The estimated K_d for the binding of [^{3}H]QNB to the transfected HM1 receptor in these cells, as assessed in saturation binding analyses, was some 200 pM. In experiments which measured the receptor population by using a single concentration of [^{3}H]QNB, this value for the K_d was used to correct for the percentage occupancy of the receptors by the [^{3}H]ligand.

Adenylate cyclase assays

These were performed as described in [23]. [^{32}P]ATP and cyclic [^{32}P]AMP were separated by the method of Salomon et al. [24].

Inositol phosphate assays

Cells were seeded in 24-well plates and labelled to isotopic equilibrium with [^{3}H]inositol (1 μCi/ml) in inositol-free Dulbecco's medium containing 1% dialysed newborn-calf serum for 48 h.

On the day of experiments, the labelling medium was removed and the cells were washed twice with 0.5 ml of Hanks buffered saline, pH 7.4, containing 1% (w/v) BSA and 10 mM glucose (HBG). The cells were incubated for 10 min with HBG containing 10 mM LiCl (HBG/LiCl), and stimulation was carried out with agonists or other agents in HBG/LiCl for 20 min. All incubations were performed at 37 °C. In some cases, cells were treated with carbachol (1 mM) during the final 16 h of labelling with *myo*-[^{3}H]inositol. Reactions were terminated by addition of 0.5 ml of ice-cold methanol. The cells in each well were then scraped and transferred to vials. Chloroform was added to a ratio of 1:2 (chloroform/methanol; v/v) and the samples were extracted overnight. The phases were split by addition of chloroform and water to final proportions of 10:10:9 (chloroform/methanol/water; by vol.), and the upper phase was taken for analysis of total inositol phosphates.

Total inositol phosphates were analysed by batch chromatography on Dowex-1 formate as previously described [25].

Immunological studies

The generation and specificities of the various antisera used in this study are shown in Table 1. Each antiserum was produced in a New Zealand White rabbit by using a conjugate of a synthetic peptide and keyhole-limpet haemocyanin (Calbiochem) as antigen. The details of this process have previously been recorded in detail [26]. Membrane samples were resolved by SDS/PAGE [10% (w/v) acrylamide] in 14 cm × 16 cm slab gels overnight at 60 V. Proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2 h at 37 °C with 5% gelatin in Tris-buffered saline (TBS) (20 mM Tris/HCl, pH 7.5, 500 mM NaCl). Primary antiserum (1:200 dilution unless otherwise noted) in 1% gelatin/TBS was then added and left overnight. The primary antiserum was then removed, and the blot was washed extensively with distilled water, followed by washes with TBS containing 0.1% (v/v) Tween 20 (TTBS) and then with TBS. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase) in 1% gelatin/TBS was added and left for 3 h. Removal of the secondary antiserum was followed by the same series of extensive washes of the nitrocellulose as detailed after removal of the primary antiserum. *o*-Dianisidine hydrochloride (Sigma) was employed as the substrate for detection of the antibody complex.

The developed immunoblots were scanned with a Shimadzu CS-9000 dual-wavelength flying-spot laser densitometer on reflectance mode at 500 nm. The results were analysed on a Shimadzu FDU-3 central processing unit, enabling quantification of the immunoblots.

RESULTS

Levels of the human M1 muscarinic acetylcholine (HM1) receptor expressed in membranes of a clone of CHO cells after selection of cells stably transfected with cDNA encoding this polypeptide were assessed in saturation binding analyses using [^{3}H]QNB to measure total binding and parallel assays which also contained atropine (10 μM) to define non-specific binding. Transformation of the specific binding data defined by these parameters by the method of Scatchard [27] (Figure 1a) indicated the presence of a single class of high-affinity receptor sites, with $B_{max.}$ 1631 ± 155 fmol/mg of membrane protein and K_d 192 ± 23 pM (means ± S.E.M., $n = 4$ for individual membrane preparations) for [^{3}H]QNB. No specific binding of [^{3}H]QNB was detected in membranes of parental untransfected CHO cells (results not shown). Displacement of specific [^{3}H]QNB binding from membranes of the HM1-receptor-expressing cells could be achieved by the muscarinic antagonist atropine, with an IC_{50} (corrected for receptor occupancy) of 1.2 nM, and by the cholinergic agonist carbachol, with an IC_{50} (corrected for receptor occupancy) of 17 μM (results not shown).

Membranes of parental CHO cells and of those expressing the HM1 receptor contained a 42 kDa polypeptide which was detected by immunoblotting with an antiserum (CQ2) which identifies the C-terminal decapeptide common to $G_q\alpha$ and G11α [28] (see Figure 3 below). Similar levels of this polypeptide were present in membranes of the parental and transfected cells. Immunological detection of this polypeptide by antiserum CQ2 in membranes derived from a variety of tissues, including CHO cells and rat brain cortex, was prevented by the presence of increasing concentrations of the peptide which was used to generate this antiserum, but not by peptides representing the equivalent region of other G-proteins (results not shown). Half-maximal effects of the competing cognate peptide were produced at 5 ng/ml. Immunoblotting of increasing amounts of membranes of HM1 expressing CHO cells with antiserum CQ2 (Figure 2a) demonstrated a linear increase in immunological

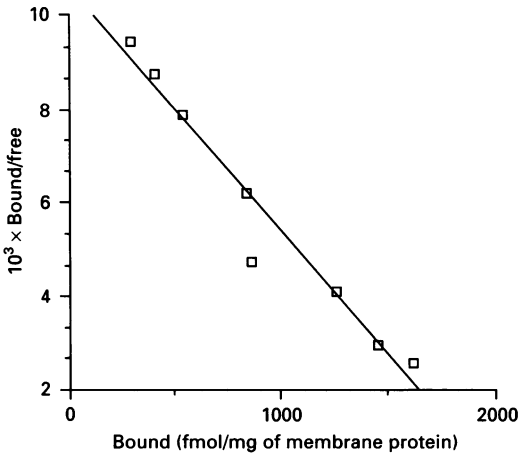

Figure 1 Expression of the HM1 receptor in CHO cells: saturation binding studies with [^{3}H]QNB

Membranes (5 μg) from HM1-expressing CHO cells were incubated with [^{3}H]QNB (50 pM–1.0 nM) in the absence or presence of atropine (10 μM) to define non-specific and total binding respectively. The specific binding estimated in this manner was transformed in accordance with Scatchard [27]. In the experiment displayed, $B_{max.}$ for [^{3}H]QNB was 1683 fmol/mg of membrane protein and the K_d of the [^{3}H]ligand for the receptor was 194 pM.

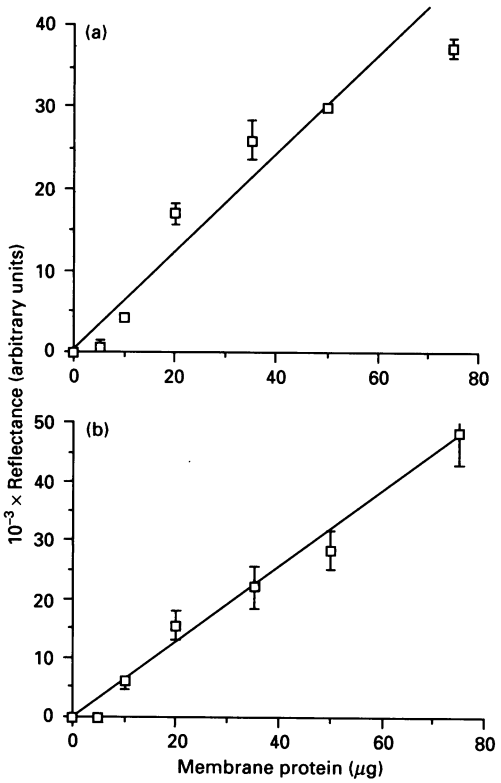

Figure 2 Detection of $G_q\alpha$/G11α by antiserum CQ2 and of $G_q\alpha$ by antiserum IQ1 in membranes of HM1-expressing CHO cells: dependence on protein amount

Different amounts of HM1-expressing CHO-cell membranes were resolved by SDS/PAGE, transferred to nitrocellulose and immunoblotted with antiserum CQ2 (**a**) or with antiserum IQ1 (**b**). The developed immunoblots were then scanned as described under 'Methods'. An essentially linear relationship between intensity of colour development and membrane amount was observed for both antisera over the range 5–75 μg of membrane protein. Results are presented as means ± S.E.M. ($n = 3$). Based on these studies, experiments designed to examine potential regulation of levels of $G_q\alpha$/G11α routinely used 25 μg of membrane protein per gel lane. Similar curves were constructed (see [11] for example) for the other antisera used in this study (see Table 1) to ensure that maximal potential to note receptor-induced regulation of other G-proteins could be recorded (results not shown).

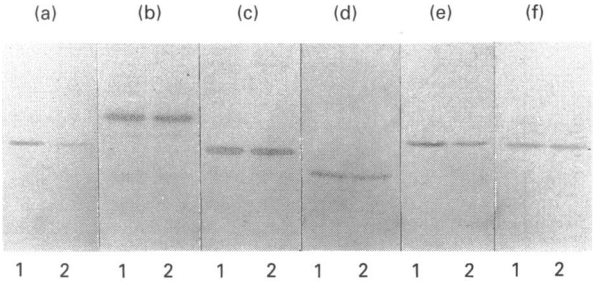

Figure 3 Carbachol treatment of HM1-expressing CHO cells decreases levels of immunologically detectable membrane-associated $G_q\alpha$, but not other G-protein α subunits

Membranes (25 μg) from untreated (1) and carbachol (1 mM, 16 h) (2)-treated HM1-expressing CHO cells were immunoblotted for (a) $G_q\alpha$/G11α with antiserum CQ2, (b) $G_s\alpha$ with antiserum CS1, (c) $G_i2\alpha$ with antiserum SG1, (d) β-subunit with antiserum BN3 and (e) $G_q\alpha$ with antiserum IQ1. Membranes (25 μg) from parental CHO cells which were either untreated (1) or treated with carbachol (1 mM, 16 h) (2) were also immunoblotted (f) for $G_q\alpha$/G11α with antiserum CQ2. See Table 2 for quantitative details.

Table 2 Agonist regulation of G-proteins in HM1-expressing CHO cells

Relative levels of $G_q\alpha$, $G_s\alpha$, $G_i2\alpha$ and G-protein β subunit were assessed by quantitative immunoblotting using the antisera defined in Table 1. Results are presented as means $\pm$ S.E.M. ($n = 4$ for antiserum SG1, 5 for antisera IQ1 and CS1, 6 for antiserum BN3 and 8 for antiserum CQ2) of levels of the individual G-protein polypeptides relative to membranes of untreated cells maintained and processed in parallel. Statistical analysis using Student's t test for paired samples indicated that the effect of carbachol treatment was significant for antisera IQ1 ($P = 0.009$) and CQ2 ($P < 0.001$). It was insignificant for antisera CS1 and SG1. Results for antiserum BN3 approached, but did not reach, statistical significance ($P = 0.06$).

G-protein	Immunologically detected G-protein (% of that in untreated cells)
$G_q\alpha$ (antiserum IQ1)	60.0 $\pm$ 8.5
$G_q\alpha$ + G11α (antiserum CQ2)	59.8 $\pm$ 3.4
$G_s\alpha$ (antiserum CS1)	88.7 $\pm$ 8.5
$G_i2\alpha$ (antiserum SG1)	97.8 $\pm$ 6.5
β-subunit (antiserum BN3)	82.5 $\pm$ 7.5

signal with membrane amounts between 5 and 75 μg. On this basis, 25 μg of membrane protein was used routinely for all subsequent studies. Similar results were obtained with antiserum IQ1 (Figure 2b), which was generated against a synthetic peptide corresponding to amino acids 119–134 of $G_q\alpha$.

When HM1-expressing CHO cells were treated with the cholinergic agonist carbachol (1 mM, 16 h) cell elongation was noted (results not shown). Despite the alteration in cellular morphology caused by carbachol treatment of the HM1-receptor-expressing CHO cells, no gross effect on the polypeptide composition of the membranes was observed. Coomassie-Blue stained gels of membranes from untreated and carbachol-treated cells were indistinguishable (results not shown). By contrast, no effect of carbachol was noted on the cellular morphology of parental CHO cells. Immunoblotting of membranes prepared from HM1-expressing CHO cells with antiserum CQ2 demonstrated a marked decrease in levels of $G_q\alpha$/G11α in the cells exposed to carbachol, in comparison with the untreated cells (Figure 3, lane a). No decrease in membrane-associated levels of $G_q\alpha$/G11α was noted in carbachol-treated parental CHO cells (Figure 3, lane f). In contrast with $G_q\alpha$, membrane-associated levels of $G_s\alpha$, $G_i2\alpha$ and $G_i3\alpha$ were not decreased by carbachol treatment of HM1-

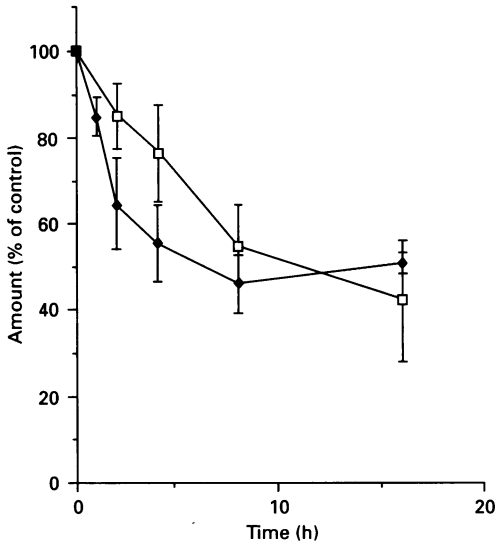

Figure 4 Concurrent time courses of down-regulation of $G_q\alpha$ and the HM1 receptor during carbachol treatment of HM1-expressing CHO cells

After treatment of HM1-expressing CHO cells with carbachol (1 mM) for various times, levels of both the HM1 receptor (□) and $G_q\alpha$ (◆) were assessed in membranes from the cells by measuring the specific binding of [^{3}H]QNB (1 nM) and by immunoblotting with antiserum CQ2.

containing CHO cells (Figure 3, Table 2, and results not shown), nor were levels of these three G-proteins modified by treatment of parental CHO cells with carbachol (results not shown).

Confirmation of the down-regulation of $G_q\alpha$ by treatment of the HM1-expressing CHO cells with carbachol was provided by immunoblotting of membranes of treated and untreated cells with antiserum IQ1. This antiserum also identified a single polypeptide of 42 kDa, which was decreased by some 40% in membranes isolated from the carbachol-treated HM1-expressing CHO cells in comparison with the untreated cells (Figure 3, lane e; Table 2). Carbachol treatment of HM1-expressing CHO cells also resulted in a down-regulation of some 20% of membrane-associated β-subunit immunoreactivity, as detected by antiserum BN3 (Figure 3, lane d; Table 2).

Carbachol-induced loss of membrane-associated $G_q\alpha$ was not a reflection of transfer of this polypeptide to the cytoplasm. Immunoblotting of total membrane and cytoplasmic fractions of untreated HM1-expressing CHO cells demonstrated that $G_q\alpha$ was barely detectable in the cytoplasmic fraction. The decrease in membrane-associated levels of $G_q\alpha$ caused by carbachol treatment was not accompanied by a corresponding increase in detection of this polypeptide in the cytoplasmic fraction (results not shown). Treatment of HM1-expressing CHO cells with carbachol (1 mM) for various times indicated that half-maximal loss of $G_q\alpha$ was produced by 3 h (Figure 4). Parallel assessment of the down-regulation of the HM1 muscarinic receptor as assessed by the decrease in the specific binding capacity for [^{3}H]QNB indicated that the time course of loss of this polypeptide was similar to that of the G-protein (Figure 4).

Dose/response curves of carbachol treatment of the HM1-expressing CHO cells indicated that half-maximal loss of the $G_q\alpha$ polypeptide was achieved by treatment with 5 μM agonist (Figures 5a and 5b). Half-maximal loss of the HM1 receptor, as assessed by the specific binding of [^{3}H]QNB to membranes of untreated and carbachol-pretreated cells, was also produced by the same concentration (5 μM) of the agonist (Figure 5b). Carbachol markedly stimulated the inositol phosphate

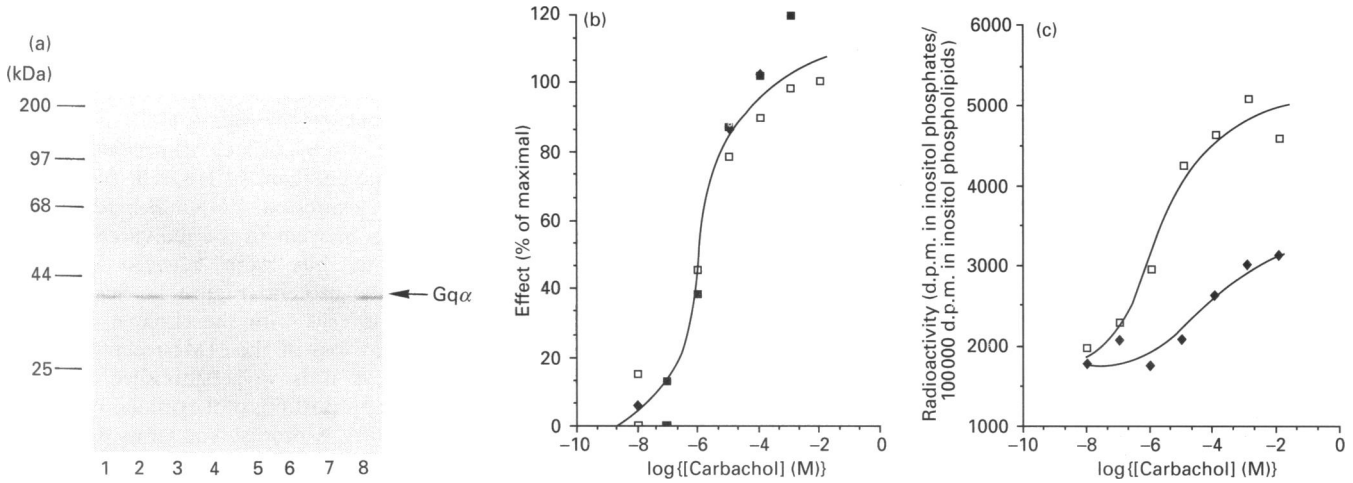

Figure 5 Dose–response curves for carbachol-induced decreases in cellular levels of the HM1 receptor and $G_q\alpha$ and for carbachol-induced generation of inositol phosphates in HM1-expressing CHO cells

(**a**) Immunological detection of $G_q\alpha$ in membranes of HM1-expressing CHO cells after treatment with carbachol. HM1-expressing CHO cells were treated for 16 h with carbachol at (1) 10 nM, (2) 0.1 μM, (3) 1 μM, (4) 10 μM, (5) 0.1 mM, (6) 1 mM or (7) 10 mM, or (8) without carbachol. Membranes (25 μg) prepared from these cells were immunoblotted as described under 'Methods' with antiserum CQ2 as primary antiserum. (**b**) Carbachol-induced down-regulation of both the HM1 receptor and $G_q\alpha$ and carbachol induced-generation of inositol phosphates in HM1-expressing CHO cells: quantitative aspects. The data from (**a**) (■) were quantified as described under 'Methods', and levels of the HM1 receptor were assessed by measuring the specific binding of [^{3}H]QNB (◆). Carbachol-induced inositol phosphate generation (□) was measured as described under 'Methods'. Data from these experiments were then plotted as percentages of the maximal effect produced by the agonist. (**c**) Carbachol-induced inositol phosphate generation in HM1-expressing CHO cells: effect of carbachol pretreatment. HM1-expressing CHO cells which were either pretreated (◆) or not (□) with carbachol (1 mM, 16 h) were subsequently stimulated with various concentrations of carbachol, and the generation of inositol phosphates was measured as described under 'Methods'. In the experiment shown, basal inositol phosphate generation (i.e. in the absence of carbachol) was 1953 ± 136 d.p.m./100000 d.p.m. in inositol phospholipids in the untreated cells and 1913 ± 165 d.p.m./100000 d.p.m. in inositol phoslipids in the carbachol-pretreated cells.

generation in LiCl-treated HM1-expressing CHO cells which had been labelled for 48 h with *myo*-[^{3}H]inositol; 5 μM carbachol half-maximally stimulated inositol phosphate generation in the HM1-expressing CHO cells (Figure 5c).

Over a 5 min incubation period, carbachol was able to generate some 3000 d.p.m. of inositol phosphates per 100000 d.p.m. in inositol-containing phospholipids above the basal levels (Figure 5c). After carbachol (1 mM) pretreatment of the cells for 16 h, subsequent challenge with different concentrations of carbachol was considerably less effective in the generation of inositol phosphates (approx. 1000 d.p.m./100000 d.p.m. in inositol-containing phospholipids) (Figure 5c), demonstrating a desensitization of the receptor-mediated response.

We assessed whether blockade of $G_q\alpha$ synthesis *de novo* would be able to mimic the down-regulation of the G-protein which was produced by carbachol treatment. HM1-expressing CHO cells were treated with cycloheximide (100 μg/ml) for 8 h in the absence or presence of carbachol (1 mM). Treatment of the cells with cycloheximide in isolation did produce a decrease in membrane-associated levels of $G_q\alpha$, by some 20 % over this time period (Table 3), but this effect was considerably smaller than the decrease in $G_q\alpha$ produced by carbachol. Furthermore, the effects of carbachol and cycloheximide on membrane levels of $G_q\alpha$ were essentially additive (Table 3). As such, inhibition of transcription could not account for the effects of carbachol on cellular $G_q\alpha$ levels, because even if transcription was completely blocked and translation of pre-existing mRNA was abolished (i.e. in the presence of cycloheximide, such that no protein synthesis *de novo* was occurring), then levels of $G_q\alpha$ would be expected to be decreased by only 20 % over an 8 h period. As a control for this experiment, the effects of cycloheximide treatment on membrane-associated levels of $G_i2\alpha$ were measured in parallel. Cycloheximide treatment for 8 h caused a loss of 30 % of immunodectable $G_i2\alpha$ (Table 3), a value very similar to that produced by the same treatment of NG108-15 cells [11]. As expected, the co-addition of carbachol did not modify the effects of cycloheximide on cellular levels of G_i2 (Table 3).

Table 3 Effect of cycloheximide on carbachol-induced loss of Gqα from HM1-expressing CHO cells

HM1-expressing CHO cells were treated with cycloheximide (100 μg) or carbachol (1 mM) or a combination of these agents. In the experiments which contained both cycloheximide and carbachol, cycloheximide was added 30 min before addition of carbachol. Membranes (25 μg) prepared from the cells were immunoblotted for either $G_q\alpha$ or $G_i2\alpha$ as described under 'Methods', and the developed immunoblots were quantified by laser densitometry. Results are presented from a single experiment. A further experiment produced similar data.

Treatment	G-protein... $G_q\alpha$ (% of untreated)	$G_i2\alpha$ (% of untreated)
Carbachol (8 h, 1 mM)	54.4	111.3
Cycloheximide (8.5 h, 100 μg/ml)	78.4	69.6
Carbachol (8 h, 1 mM) + cycloheximide (8.5 h, 1 mM)	31.7	67.6

DISCUSSION

Clear evidence now indicates that receptor regulation of phosphoinositidase C activity is transduced by members of the G_q family of heterotrimeric G-proteins (4,5,29,30]. On the basis of availability of information on cDNA corresponding to the α subunits [6–8] of these G-proteins, it has been possible to generate antisera able to identify these polypeptides selectively. Such an approach has provided the first convenient means of detection of

these polypeptides, as covalent incorporation of [^{32}PADP-ribose cannot be achieved in a similar manner to that for the α subunits of G_s and of the 'G_i-like' G-proteins, as the G_q-like G-proteins are not modified by either cholera toxin or pertussis toxin. The use of such antisera has confirmed the expected widespread distribution of G_q [28,29], and now allows assessment of whether regulation of cellular levels of G_q occurs.

Hormonally controlled regulation of cellular G-protein levels have been recorded in several situations. For example, dexamethasone treatment of pituitary GH3 cells has been reported to result in increased levels of $G_s\alpha$ mRNA, protein and function [31], and it is firmly established that in animal models of hypothyroidism there are elevated levels of the 'G_i-like' G-proteins in membrane fractions isolated from a range of tissues [32–34]. For both glucocorticoid and thyroid hormones it would be expected that these effects would be produced at the level of gene transcription.

In several other instances it has been reported that chronic exposure of cells to agonists which function at G-protein-linked receptors can also lead to alterations in cellular levels of G-proteins. In some of these cases the observed effect is principally one of 'cross-regulation', i.e. activation of a receptor alters the cellular levels of a G-protein which is not that which the receptor uses to regulate its effector system(s). It has been noted in this regard that agonist activation of the β-adrenergic receptor of S49 lymphoma cells can result in a substantial up-regulation of $G_i2\alpha$ [14]. As G_i2 has been shown to act as the inhibitory regulator of adenylate cyclase in a number of systems [15,16], then this effect of β-receptor activation, which can be mimicked by the generation of cyclic AMP by receptor-independent means, results in more effective inhibition of adenylate cyclase and thus seems to provide one means for cellular adaptation to limit the effects of sustained β-receptor occupancy.

In some other cases, however, the G-protein(s) which are altered in amount in the cell in response to agonist–receptor interaction are those which might be expected to be activated by this process. For example, treatment of rat white adipocytes, which are maintained in tissue culture, with (−)-N^6-phenylisopropyladenosine, which is an adenosine deaminase-resistant analogue of adenosine, causes a marked decrease in membrane levels of the α subunits of each of the pertussis-toxin-sensitive G-proteins G_i1, G_i2 and G_i3 by activating an adenosine A1 receptor [12]. Such treatment has no effect on cellular levels of either the 43 or 47 kDa forms of the α subunit of G_s which are expressed by these cells [12].

Such effects are not restricted to the pertussis-toxin-sensitive G-proteins. We have reported that treatment of NG108-15 neuroglioma cells with either prostaglandin E_1 (which in this system causes stimulation of cyclic AMP production) [11] or with iloprost (a stable analogue of prostacyclin) [35–36] causes a marked down-regulation of cellular levels of $G_s\alpha$. Levels of the α subunits of G_i2, G_i3 and G_o were unaffected by such treatment [11]. Others have also recorded prostanoid-receptor-mediated decrease is $G_s\alpha$ in these cells [37]. We have also noted that the time courses and dose–response curves for prostanoid-agonist-mediated loss of G_s and of the prostanoid receptor are coincident, and on this basis suggested that co-down-regulation of receptor and G-protein may occur [35,36]. Furthermore, the stoichiometry of loss of receptor and G-protein is approximately one receptor to eight G-proteins [35], a number similar to the estimation of stoichiometry of activation of G_s by β-adrenergic receptors in reconstitutive assays [38].

Given this experimental framework, the present studies were undertaken to assess potential regulation of cellular G-protein complement after activation of a receptor established to function via the activation of a phosphoinositidase C. The system that we have used is a CHO cell transfected with cDNA encoding the HM1 receptor, and as such the non-transfected parental cell line served as an appropriate control for the effects which we record, as the parental cell does not express detectable levels of any of the muscarinic receptors, as defined by the lack of specific binding of the non-selective muscarinic antagonist [^{3}H]QNB. By contrast, the transfected cell line expressed [^{3}H]QNB-binding sites. Numbers of these varied somewhat in the individual passages used for the experiments, but were between 1300 and 2000 fmol/mg of membrane protein (Figure 1). Sustained receptor occupation of these cells with the cholinergic agonist carbachol caused a marked loss of the HM1-receptor-binding site. This was expected, as it is well established that such treatments cause the loss of proportions of the cellular population of G-protein-linked receptors, a process known as down-regulation [9]. However, in parallel with the loss of HM1 receptors, we have noted an equally marked loss of immunologically detectable membrane levels of $G_q\alpha$. Such a conclusion is based on the immunological identification of an apparently single 42 kDa polypeptide by two separate antisera. Antiserum CQ2 was raised against the C-terminal decapeptide which is common to $G_q\alpha$ and $G11\alpha$, and as such cannot be used to discriminate between these two members of the G_q family of G-proteins [28]. However, it is a highly useful probe as it shows no cross-reactivity [28,39] with members of the G_s, G_i and G13 families [40] of G-proteins and also shows no detectable cross-reactivity in e.l.i.s.a. assays with a peptide corresponding to the equivalent region of G16 [39].

Antiserum IQ1 was generated against an internal region of $G_q\alpha$ (amino acids 119–134), which is substantially different in $G_q\alpha$ and $G11\alpha$. We do not at present have an antiserum which would allow unambiguous detection of $G11\alpha$; thus, although it is clear that agonist activation of the HM1 receptor in these cells results in a substantial down-regulation of $G_q\alpha$, it remains to be examined clearly whether the same is true of $G11\alpha$. In contrast with $G_q\alpha$, immunologically detected levels of the α subunits of G_s, G_i2 and G_i3 were unaffected by carbachol treatment of the cells (Figure 3, Table 2). Requirement for the activation of the HM1 receptor to produce cellular loss of $G_q\alpha$ is clear, as equivalent treatment of parental untransfected CHO cells with carbachol had no effect on cellular levels of $G_q\alpha$ or any other G-protein that we have been able to assess. The decrease in membrane-associated $G_q\alpha$ levels after carbachol treatment of the HM1-expressing CHO cells was not a simple reflection of transfer of the polypeptide from membrane to cytosol, as seems to be the case for $G_s\alpha$ after treatment of S49 lymphoma cells with β-adrenergic-receptor agonists [41]. Within the limits of the immunological assay, we were unable to detect any increase in cytosolic levels of $G_q\alpha$ during time courses of treatment of HM1-expressing CHO cells with carbachol (results not shown). Down-regulation of HM1 receptors and $G_q\alpha$ in the receptor-expressing cells required similar concentrations and times of exposure to carbachol, suggesting that the loss of these two polypeptides may well be linked. Furthermore, the concentration of carbachol required to produce half-maximal down-regulation of these polypeptides was similar both to that required to half-maximally stimulate inositol phospholipid hydrolysis and to that estimated to produce half-maximal receptor occupancy. Such results indicate that receptor occupancy is strictly required to observe agonist induced loss of $G_q\alpha$.

The kinetics and extent of down-regulation of receptor and G-protein, although similar, were not identical (Figure 4). However, observed degrees of down-regulation may not be identical for polypeptides which are co-internalized, as such an observation

would require that the rates of turnover of the two proteins be similar and that no other alterations in the balance of synthesis and degradation were produced in parallel. This may not be true. It has recently been noted that, in cells co-expressing β1 and β3 adrenoceptors, long-term exposure to the agonist isoprenaline results in down-regulation of the β1 receptor but an apparently paradoxical up-regulation of β3 receptors [42]. This reflects the fact that the promoter for the β3 receptor gene contains a number of cyclic-AMP-responsive elements, and thus activation of the receptor leads to enhanced transcription of the gene, which in this case more than compensates for the agonist-induced down-regulation [42]. Most models of receptor–G-protein interaction suggest that a physical dissociation of the components will occur after agonist-promoted guanine nucleotide exchange on the α subunit of the G-protein, and it is also widely thought that receptor internalization and down-regulation occur independently of down-regulation of the G-protein. However, a recent study of mutant forms of the β2 adrenergic receptor has indicated that forms of the receptor which interact poorly with the G-protein signalling system, as assessed by the signal that they are able to generate, are also impaired in their down-regulation patterns after exposure to agonist [43]. Furthermore, agonist-induced down-regulation of the β2 receptor, although demonstrating a requirement for interaction of receptor and G-protein, is not dependent on the generation of cyclic AMP [43,44]. Such an observation is entirely in accord with our studies, which have shown that prostanoid-receptor-mediated down-regulation of G_s is independent of cyclic AMP production [11]. Such studies on mutated receptors, in concert with the studies reported herein, may suggest that it is the receptor–G-protein complex which is the target for processing for down-regulation.

These studies demonstrate for the first time agonist-induced alteration in cellular levels of $G_q\alpha$ and provide further evidence that receptors which activate phosphoinositidase C (in this case the HM1 receptor) interact directly with G_q.

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