Down-regulation of the G-proteins $G_q\alpha$ and $G_{11}\alpha$ by transfected human M_3 muscarinic acetylcholine receptors in Chinese hamster ovary cells is independent of receptor down-regulation

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Chinese hamster ovary cells stably transfected with human M_3 muscarinic acetylcholine receptors show a 40–50% reduction in the immunoreactive G-proteins $G_q\alpha$ and $G_{11}\alpha$ when stimulated with the cholinergic agonist carbachol. This effect is seen after 9 h, is maximal after 24 h, and occurs over a range of carbachol concentrations that activate phosphoinositide hydrolysis in these cells. The effect is specific for $G_q\alpha$ family proteins as $G_s\alpha$ was slightly increased after carbachol treatment and $G_{i3}\alpha$ was unchanged. Using a urea gel system, we were able to resolve $G_q\alpha$ and $G_{11}\alpha$, both of which were down-regulated by carbachol. An M_3 receptor mutant, with C-terminal threonines changed to alanines as described previously, binds ligand and activates phosphoinositide hydrolysis normally but is not down-regulated in response to carbachol. This receptor, however, induces $G_q\alpha/G_{11}\alpha$ down-regulation similarly to wild-type M_3 receptors, indicating that G-protein down-regulation is not directly coupled to receptor down-regulation. Thus down-regulation of $G_q\alpha$ and $G_{11}\alpha$ may contribute to heterologous desensitization particularly at longer times of agonist exposure.

INTRODUCTION

Prolonged exposure of a cell to an agonist that binds to a cell surface receptor usually results in a decrease in the total number of cellular receptors for that agonist [1]. This process, termed receptor down-regulation, usually results from increased rates of receptor internalization and degradation. Recently it has been found that down-regulation of the seven-transmembrane domain class of membrane receptors is often accompanied by down-regulation of the G-protein activated by the receptor [2]. This was first studied using receptors that regulate adenylate cyclase through members of the G_s and G_i G-protein families [3–6]. Furthermore, the time course and dose–response for G-protein and receptor down-regulation were similar. This led to the speculation that down-regulation of the G-protein and the receptor were related [2].

More recently this work has been extended to the M_1 muscarinic acetylcholine receptor which, when expressed in Chinese hamster ovary (CHO) cells, was observed to undergo ligand-induced down-regulation of both the receptor and G-proteins of the G_q class [7]. This was subsequently shown to be the result of enhanced degradation of these G-proteins and to affect both $G_q\alpha$ and $G_{11}\alpha$ to the same extent [8,9]. We have performed studies of M_3 muscarinic acetylcholine receptors expressed in CHO cells and identified threonine residues in the C-terminal domain that do not affect intracellular signalling but are necessary for agonist-induced receptor down-regulation [10]. We have now used the cell lines expressing these receptors to test whether the M_3 muscarinic acetylcholine receptor will also down-regulate G-proteins and whether the down-regulation of the G-protein is dependent on down-regulation of the receptors. The data demonstrate that M_3 receptor activation down-regulates both $G_q\alpha$ and $G_{11}\alpha$ and that this effect is independent of receptor down-regulation.

EXPERIMENTAL

Materials

Antiserum QL to a common region present in $G_q\alpha$ and $G_{11}\alpha$, and antiserum RM/1 specific for $G_s\alpha$ were obtained from DuPont–New England Nuclear (Boston, MA, U.S.A.); antiserum C-10 to $G_{i3}\alpha$ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). *N*-[^{3}H]Methylscopalamine (NMS) (81.5 Ci/mmol) was from New England Nuclear. Human embryonic kidney (HEK 293) cells stably overexpressing $G_q\alpha$ and $G_{11}\alpha$ [11] were obtained from Arieh Katz (Pasadena, CA, U.S.A.). Materials for gel electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.), and material for tissue culture was from Gibco (Grand Island, NY, U.S.A.). Nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH, U.S.A.). Detection reagents for enhanced chemiluminescence and horseradish peroxidase-coupled anti-rabbit antibody were from Amersham Corp. (Arlington Heights, IL, U.S.A.).

Cell culture and preparation of cell membranes

CHO-K1 cells were originally obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). They were stably transfected with wild-type human M_3 muscarinic acetylcholine receptor or a mutant receptor in which threonine residues 550, 553 and 554 had been converted into alanine by site-directed mutagenesis [10]. Wild-type clone 14 and the mutant expressed similar numbers of receptors (approx. 150000/cell) as assessed by [^{3}H]NMS binding. Cells were routinely grown in 10 cm Petri dishes and membranes were prepared from individual dishes shortly after the cells became confluent. After being washed with PBS, cells were scraped into 1 ml of 20 mM Tris/HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EDTA, 0.3 mM EGTA, 0.05% bacitracin and 0.01% soya-bean trypsin inhibitor. Cells

Abbreviations used: CHO, Chinese hamster ovary; NMS, *N*-methylscopalamine; HEK, human embryonic kidney.
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were frozen and thawed three times in solid CO_2/ethanol and then passed 10 times through a 27-gauge needle. The homogenate was centrifuged at 200 *g* for 5 min at 4 °C to remove nuclei and debris after which membranes were pelleted from the supernatant at 43000 *g* for 30 min in a Beckman Ti 70.1 rotor. The pellet was resuspended in 100 μl of Tris/HCl buffer. After determination of protein content with a Bio-Rad protein assay kit, the membranes were solubilized in Laemmli sample buffer with 2% 2-mercaptoethanol [12] by boiling for 5 min. Membranes were similarly prepared from HEK 293 cells and other cell lines.

Receptor binding and down-regulation

Receptor-binding assays were carried out on membranes prepared as described above. For saturation binding studies 0.03–2 nM concentrations of the radioactive antagonist, [^{3}H]NMS, were used. For the study of receptor down-regulation, membranes were prepared after cells had been preincubated with 1 mM carbachol for 24 h at 37 °C. The concentration of the radioligand used for down-regulation binding studies was 2 nM. Membranes were incubated in 1 ml of buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose and 30 mM Hepes buffer, pH 7.4). Non-specific binding was determined in the presence of 10 μM atropine. Binding assays were performed at room temperature for 3 h. Final membrane protein concentrations were 20 μg/ml. The binding reactions were terminated by filtration on to Whatman GF/C filters followed by washing four times with 5 ml of ice-cold 0.9% NaCl. Radioactivity was determined in 5 ml of Bio-Safe II scintillation fluid (Research Products International, Mount Prospect, IL, U.S.A.). Saturation binding data were analysed by a radioligand-binding analysis program adapted for the IBM PC (Elsevier-BIOSOFT, Cambridge, U.K.).

Immunological analysis

Membrane samples were routinely resolved by SDS/PAGE using 10% (w/v) polyacrylamide slab gels (14 cm × 16 cm × 1.5 mm) over 4–5 h of constant current application. CHO cell membrane protein (50 μg) or HEK 293 cell membranes (25 μg) was routinely used. When specified, urea gradient gels were used to resolve $G_q\alpha$ and $G_{11}\alpha$ [9,13]. This system utilized 9% (w/v) acrylamide, 0.26% (w/v) bisacrylamide containing a linear urea gradient of 4–8 M at pH 6.8. Samples were electrophoresed at 150 V for 8 h with cooling at 8 °C in a Bio-Rad Protean II apparatus. After electrophoresis, proteins were transferred overnight at 30 V to nitrocellulose membranes as described by Towbin et al. [14] using glycine/Tris/methanol transfer buffer containing 0.1% SDS as reported previously [15]. Nitrocellulose membranes were blocked with Tris-buffered saline (TBS) containing 137 mM NaCl, 20 mM Tris/HCl (pH 7.6), 0.3% Tween 20 and 10% ovalbumin for 1 h. All subsequent steps were carried out using TBS buffer with 3% BSA instead of ovalbumin. Membranes were washed and then probed with antisera (dilutions: QL, 1:10000; RM/1, 1:5000; C-10, 0.1 μg/ml) for 1 h at room temperature. Horseradish peroxidase-labelled goat anti-(rabbit IgG) was used at 1:5000 dilution for 1 h followed by detection with enhanced chemiluminescence reagent as specified by the manufacturer. Membranes were exposed to Kodax X-Omat AR-5 film for visual presentation and to a light-sensitive storage screen (Bio-Rad Molecular Imager) for quantification. The storage screen was then scanned using a moveable laser and the data were processed using software provided by the manufacturer. Mean band density was usually expressed as a percentage of that recorded from control membranes. For multiple analysis, membranes were stripped by heating to 50 °C for 30 min in a Tris-buffered solution containing 2% (w/v) SDS and 100 mM 2-mercaptoethanol. Control experiments in which the enhanced chemiluminescence reagent was reapplied showed the effectiveness of this procedure.

RESULTS

Our previous work on CHO cells stably expressing M_3 muscarinic receptors had shown that wild-type M_3 receptors were down-regulated after prolonged exposure to carbachol whereas cells expressing a mutant M_3 receptor with C-terminal threonines

Table 1 Effect of carbachol treatment on expression of wild-type and mutant M_3 receptors in CHO cell membranes

CHO cells expressing wild-type or mutant M_3 receptors with alanine at positions 550, 553 and 554 were incubated with or without 1 mM carbachol for 24 h. Membranes were used for binding studies with increasing concentrations from 0.03 to 2 nM [^{3}H]NMS to determine K_d and B_{max}. Samples from individual dishes were used for binding studies with a saturating concentration of ligand (2 nM) to determine down-regulation; these membranes were then solubilized, electrophoresed and analysed by Western blot with antisera QL, RM/1 and C-10. In each experiment duplicate or triplicate dishes were used to establish the level of expression in control and carbachol-treated cells, and from this the change (percentage of the control) caused by carbachol treatment was calculated. Data shown are means ± S.E.M. of four experiments.

	Receptor binding		Down-regulation by carbachol (% of control)			
	K_d (pM)	B_{max} (fmol/mg)	Receptor binding	G_q/G_{11}	$G_s\alpha$	$G_{i3}\alpha$
Wild-type	102 ± 13	947 ± 22	61 ± 1	45 ± 6	105 ± 4	91 ± 10
Mutant	90 ± 14	959 ± 72	104 ± 4	46 ± 2	116 ± 4	95 ± 13

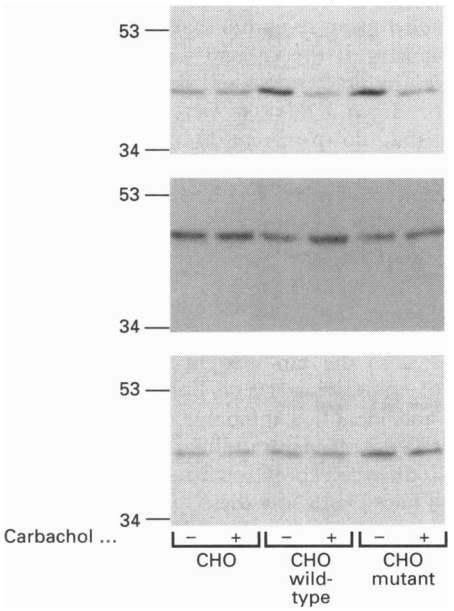

Figure 1 Western-blot analysis of G-protein α-subunits in membranes of CHO cells, cells expressing wild-type M_3 muscarinic acetylcholine receptor and mutant receptor with threonines 550, 553 and 554 changed to alanine

The top blot was probed with antiserum QL which reacts with a common sequence in $G_q\alpha$ and $G_{11}\alpha$. The middle blot was probed with antiserum RM/1 which reacts with $G_s\alpha$. The bottom blot was probed with antiserum C-10 which reacts with $G_{i3}\alpha$.

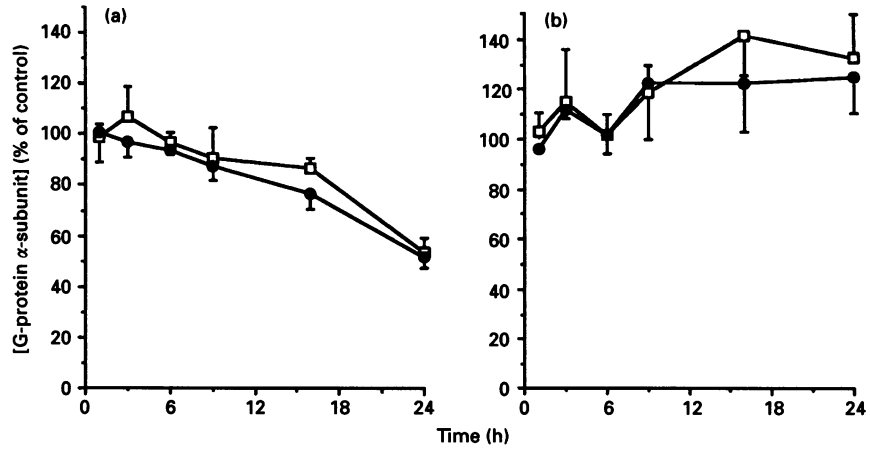

Figure 2 Time course of carbachol-induced changes in G-protein α-subunit levels probed with antisera QL (a) and RM/1 (b)

Results are means ± S.E.M. for four experiments in each of which the density of bands on Western blots was compared with the control band without carbachol treatment. Membranes were prepared from cells expressing wild-type (□) and mutant (●) M_3 muscarinic acetylcholine receptors.

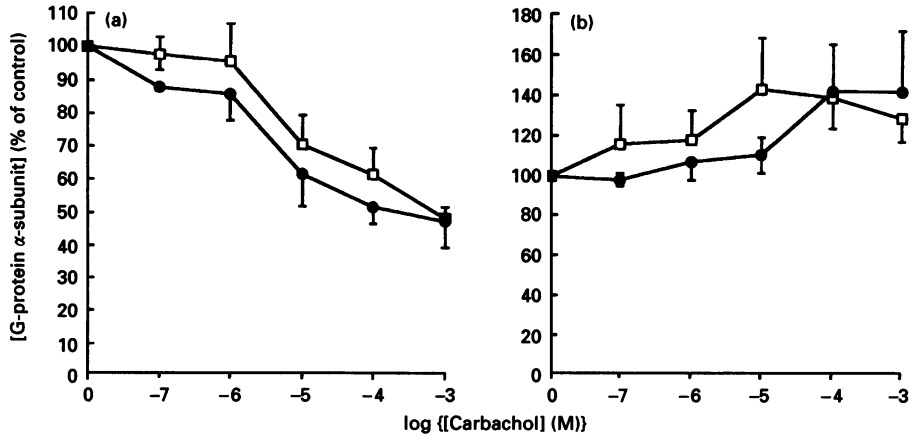

Figure 3 Concentration-dependence of carbachol-induced changes in G-protein α-subunits probed with antisera QL (a) and RM/1 (b)

Results are means ± S.E.M. of four to six experiments using membranes prepared from cells expressing wild-type (□) and mutant (●) M_3 muscarinic acetylcholine receptors.

changed to alanine showed little down-regulation [10]. Because we wished to carry out G-protein immunoblots on a membrane fraction, we first established the ligand-binding properties of M_3 receptors in CHO cell membranes. Saturation binding studies showed that membranes from cells bearing either wild-type or mutant receptor displayed a single [^{3}H]NMS-binding site with K_d approx. 100 pM and binding capacity about 950 fmol/mg of protein (Table 1). These parameters are similar to those previously measured on intact CHO cells [10]. When cells were stimulated with 1 mM carbachol for 24 h, binding to membranes bearing wild-type receptors was decreased to 61% of control whereas binding to membranes bearing mutant receptors was unchanged (Table 1). This decrease induced by carbachol is slightly less than previously reported for NMS binding in intact cells but similar to results with [^{3}H]scopolamine in intact cells [10] which also measures the total complement of cellular receptors.

Immunoblotting of CHO cell membranes (Figure 1) with antiserum QL revealed a single band at 42–43 kDa that was similar in control and transfected CHO cells. Antiserum RM/1 revealed a major band at approx. 48 kDa and a minor band at 45 kDa, which does not show on the photograph, consistent with two normal splice variants of $G_s\alpha$. The larger form was significantly more abundant and was used for subsequent quantification. Antiserum C-10 revealed a single band at 41 kDa. When cells were treated for 24 h with 1 mM carbachol there was a clear decrease in signal with antiserum QL in cells expressing wild-type receptor and those expressing the mutant receptor. There was no significant decrease in signal in membrane from native CHO cells which do not express muscarinic receptors or when any of the cell lines were evaluated for $G_s\alpha$ for $G_i\alpha$ (Figure 1).

When the dependence on the length of carbachol exposure was evaluated, there was no effect on $G_q\alpha/G_{11}\alpha$ at early time points (1–6 h); a statistically significant effect was seen only after 9 h and the maximum effect was observed at 24 h (Figure 2a). After 9 h, carbachol induced a variable increase of 10–30% in $G_s\alpha$ which was not statistically significant in any single experiment. However, when all data were pooled in which CHO cells were exposed to 1 mM carbachol for 24 h, an increase in $G_s\alpha$ expression to 117 ± 6% of control was observed in cells expressing

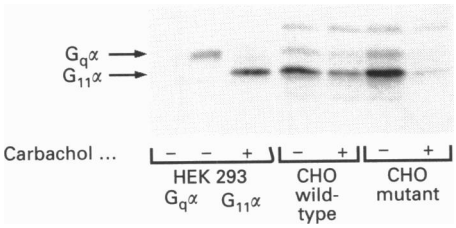

Figure 4 Down-regulation by carbachol of both $G_q\alpha$ and $G_{11}\alpha$ in CHO cells expressing wild-type and mutant M_3 muscarinic acetylcholine receptors

Proteins were resolved in an SDS/PAGE urea gradient gel system, blotted and probed with antiserum QL. The positions of $G_q\alpha$ and $G_{11}\alpha$ were identified using membranes from HEK 239 cells overexpressing these proteins.

wild-type receptor and $128 \pm 9\%$ in cells expressing the mutant receptor (both $n = 9$, $P < 0.01$ by one-tailed t test compared with 100%). There was no difference in time course of effects in cells expressing wild-type and mutant receptors. When the concentration-dependence of carbachol was evaluated (Figure 3), the mutant was slightly more sensitive, with the curve shifted about 3-fold to the left. The maximal effect of carbachol, however, observed at 1 mM was similar for membranes from the two cell types. Again, the expression of $G_s\alpha$ showed a tendency to increase in response to carbachol.

Because standard SDS/PAGE cannot resolve $G_q\alpha$ and $G_{11}\alpha$, we utilized a urea gradient to separate these species and confirmed their identity using HEK 293 cells selectively overexpressing $G_q\alpha$ and $G_{11}\alpha$ (Figure 4). In this system $G_{11}\alpha$ shows a higher mobility and, as previously reported by Mullaney et al. [9], CHO cells expressed about 2.5-fold more $G_{11}\alpha$ than $G_q\alpha$. Treatment of CHO cells with 1 mM carbachol for 24 h reduced both $G_{11}\alpha$ and $G_q\alpha$ immunoreactivity in cells expressing either wild-type or mutant receptor.

DISCUSSION

The results of the present study show that carbachol initiates down-regulation of $G_q\alpha$ and $G_{11}\alpha$ in CHO cells expressing functional M_3 receptors. M_3 receptors are known to activate phospholipase C cleavage of polyphosphoinositides with generation of diacylglycerol and inositol trisphosphate which respectively activate protein kinase C and mobilize stored intracellular Ca^{2+}. Receptors that act in this manner activate G-proteins of the G_q family whose α-subunits include q, 11, 14, 15 and 16 [16]. Of these, $G_{15}\alpha$ and $G_{16}\alpha$ are limited to haematopoietic cells whereas $G_q\alpha$, $G_{11}\alpha$ and $G_{14}\alpha$ are widely distributed [17]. Not much is known about $G_{14}\alpha$, but the development of antisera against the common C-terminal region of $G_q\alpha$ and $G_{11}\alpha$ has led to the demonstration of their presence in a number of cell types and their role in mediating polyphosphoinositide hydrolysis [18,19]. The present results confirm that CHO cells express higher levels of immunoreactive $G_{11}\alpha$ than $G_q\alpha$. This probably reflects a true difference in protein content as the sequence recognized by the antibody is identical in the two molecules, and other cells have been evaluated, including RIN cells, that show much higher levels of $G_q\alpha$ (E. van de Westerlo and J. A. Williams, unpublished work). The expression of $G_{14}\alpha$ in CHO cells has not been observed.

The down-regulation of $G_q\alpha$ and $G_{11}\alpha$ by carbachol in cells expressing M_3 receptor is qualitatively similar to results obtained for CHO cells expressing M_1 receptors [9]. In both cases the G-protein level was reduced to about 50% and was specific to members of the G_q family, as no effect was seen on G_s. G-protein regulation induced by the M_1 receptor reported by Mullaney et al. [7] was faster, with half-maximal effects reached after 4 h, whereas a significant effect of M_3 receptor activation was only observed after 9 h. Both M_1 and M_3 receptors showed parallelism between concentrations of carbachol that induced G-protein down-regulation, receptor down-regulation and phosphoinositide hydrolysis [7,10]. The fact that cells bearing the down-regulation-resistant mutant are somewhat more sensitive may be due to the reduced desensitization observed with this receptor [20].

Little is yet known about how receptor occupancy leads to selective G-protein down-regulation. Although not studied in the present work, the work of Mitchell et al. [8] clearly shows that down-regulation of $G_q\alpha$ and $G_{11}\alpha$ is mediated by accelerated degradation rather than decreased synthesis. We measured $G_q\alpha/G_{11}\alpha$ in cellular membranes, but it is possible that the α-subunits were released into the cytoplasm. However, no $G_q\alpha/G_{11}\alpha$ could be observed in cytosol prepared from control or down-regulated CHO cells, although it was present in cytosol from over-expressing HEK 293 cells (results not shown). Previous work on cells bearing M_1 receptor suggested that receptors and G-proteins were down-regulated in parallel, possibly indicating a model in which receptors and associated G-protein enter a lysosomal compartment and are degraded. However, we found normal G-protein down-regulation using the alanine mutant receptor. This receptor construct has previously been shown not to be down-regulated in CHO cells [10] or sequestered in HEK 293 cells [20]. Thus the critical step appears to be that the receptor must activate the G-protein for it to be down-regulated. This probably means that M_3 receptors activate both $G_q\alpha$ and $G_{11}\alpha$ in CHO cells. Both of these G-proteins have recently been shown to couple thyrotropin-releasing hormone and α-adrenergic receptor to phosphoinositide phospholipase C activity [11,21].

At present, the functional consequences of a 50% decrease in $G_q\alpha$ and $G_{11}\alpha$ are not known. Since prolonged stimulation by carbachol is known to desensitize cell activation by multiple mechanisms, it is tempting to speculate that G-protein down-regulation is one. Although receptor down-regulation would clearly result in homologous desensitization, the down-regulation of $G_q\alpha$ and $G_{11}\alpha$ might affect signalling by other ligand receptors that activate the same G-protein. For example, in pancreatic acinar cells, which bear M_3 muscarinic, cholecystokinin A and neuromedin C receptors, carbachol is known to desensitize the response to cholecystokinin and bombesin, i.e. heterologous desensitization. Although this could involve protein kinase C-mediated receptor phosphorylation, it may also involve G-protein down-regulation.

In summary, the present work demonstrates that prolonged stimulation of M_3 receptors in CHO cells leads to down-regulation of the G-proteins, $G_q\alpha$ and $G_{11}\alpha$, and this is independent of receptor down-regulation.

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