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The human muscarinic M1 acetylcholine receptor, when expressed in CHO cells, activates and downregulates both $G_q\alpha$ and $G_{11}\alpha$ equally and non-selectively

Ian Mullaney[a], Fiona M. Mitchell[a], J. Fraser McCallum[a], Noel J. Buckley[b] and Graeme Milligan[a]

[a]*Molecular Pharmacology Group, Departments of Biochemistry and Pharmacology, University of Glasgow, Glasgow G12 8QQ, Scotland, UK* and [b]*National Institute for Medical Research, Mill Hill, London NW7 1AA, England, UK*

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CHO cells express both of the phosphoinositidase C-linked G-proteins G_q and G_{11}. $G_{11}\alpha$ is some 2.5-fold more highly expressed than $G_q\alpha$ in membranes of these cells. Following transfection and stable expression of CHO cells with DNA encoding the human muscarinic M1 acetylcholine (HM1) receptor, chronic treatment of the cells with the cholinergic agonist carbachol resulted in down-regulation of membrane levels of both $G_q\alpha$ and $G_{11\alpha}$. Dose–response curves to carbachol produced identical EC_{50} values for agonist-induced down-regulation of the two G-proteins and both were down-regulated with the same time course. These data indicate that the HM1 receptor interacts with and activates both $G_q\alpha$ and $G_{11}\alpha$ equivalently and non-selectively in a whole cell system in which the receptor has access to both G-proteins.

Guanine nucleotide binding protein; Phospholipase C; Inositol trisphosphate; Muscarinic acetylcholine receptor

1. INTRODUCTION

Agonist regulation of cellular phosphoinositidase C activities appear to involve at least two separate mechanisms [1]. In one situation, the receptor interacts with and activates a pertussis toxin-sensitive heterotrimeric G-protein and by so doing liberates $\beta\gamma$ complex which is then able to activate the enzyme phospholipase Cβ2 and/or phospholipase Cβ3 [2–4]. In the more common situation, the receptor activates one or more members of the G_q family of G-proteins [5]. The GTP-ligated α subunits of at least G_q, G_{11} and G_{14} are then able to interact with and activate phospholipase Cβ1 [6,7].

Because of the very marked sequence similarity of G_q and G_{11} [8] it has been difficult to adequately purify these two G-proteins away from each other [9,10] and thus traditional reconstitution approaches have not been able to indicate whether a receptor is able to selectively interact with one or other of these polypeptides. Studies involving transient transfection assays have clearly indicated the potential for each of $G_q\alpha$ and $G_{11}\alpha$ to act as activators of inositol phosphate generation [6,7] but again have been unable to examine the potential selectivity of receptors for each of these G-proteins. We have recently noted in a number of systems that activation of a G-protein-linked receptor can result in down-regulation of membrane levels of the G-protein(s) which is activated by that receptor without equivalent effects on other G-proteins [11]. In CHO cells which have been transfected to express the HM1 receptor we have noted agonist-mediated down-regulation of some combination of G_q and G_{11} [12] and demonstrated that the mechanism behind this effect is enhanced turnover of the G-protein [13].

However, previously we have been unable to define if this effect involves both G_q and G_{11} and if so whether the effect of the receptor on the two G-proteins is equivalent. In this report we utilize SDS-PAGE systems which can resolve these two G-proteins [14,15] to examine this question. We demonstrate that G_q and G_{11} are both down-regulated to a similar extent following carbachol treatment of these cells and that the agonist has similar EC_{50} values for the effects on both G-proteins. These data demonstrate that in this in vivo system the HM1 receptor activates and subsequently down-regulates both G_q and G_{11} equally and non-selectively.

Correspondence address: G. Milligan, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, UK. Fax: (44) (41) 330 4620.

2. MATERIALS AND METHODS

2.1. *Cell growth*

HM1 expressing CHO cells [16] were grown in tissue culture as described previously [12]. In a number of cases the cells were incubated in the presence of varying concentrations of the cholinergic agonist carbachol prior to cell harvest. Following harvest the cells were homogenised with 10 vols. of 10 mM Tris-HCl, 0.1 mM EDTA pH 7.5 (buffer A) and the homogenates centrifuged at $500 \times g$ for 10 min. The supernatants from this process were further centrifuged at $48{,}000 \times g$ for 10 min and the pellets from the second centrifugation were washed with buffer A and recentrifuged at $48{,}000 \times g$ for 10 min. Finally the pellets were resuspended in buffer A at a protein concentration of 2

mg/ml and stored at −80°C. Protein concentration was measured according to Lowry et al. [17].

2.2. *Immunological analysis*

Membranes were resolved by SDS-PAGE in 14 × 16 cm slab gels using either the 13% (w/v) acrylamide, 0.25% (w/v) bisacrylamide system, as described by Blank et al [14] or in 12.5% (w/v) acrylamide, 0.0625% bisacrylamide containing a linear (4–8 M) gradient of urea [15] to resolve $G_q\alpha$ and $G_{11}\alpha$. Purified G_q/G_{11} from bovine liver was a kind gift of Dr. J. Exton, Vanderbilt University, TN, USA. Proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2 h at 37°C with 5% gelatin in phosphate-buffered saline (PBS) (4 mM Na_2HPO_4, 0.27 mM KCl, 0.15 mM KH_2PO_4, 137 mM NaCl) Antiserum CQ3 was generated against a synthetic peptide which represents the C-terminal decapeptide which is common to the α subunits of G_q and G_{11} [18]. Antiserum IQB was generated in a similar fashion against a synthetic peptide corresponding to amino acids 119–134 of $G_q\alpha$. This region of the protein differs considerably from the equivalent region of both $G_{11}\alpha$ and $G_{14}\alpha$. Primary antisera (CQ3, IQB) (1:1,000 dilution) in 1% gelatin-PBS were then added and left overnight. The primary antiserum was then removed and the blot was washed extensively with distilled water followed by washes with PBS containing 0.2% (v/v) NP40 and then PBS. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase) (Scottish Antibody Production Unit, Carluke, Scotland) in 1% gelatin-PBS 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 following 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. Background was subtracted by scanning of equivalent sized areas of nitrocellulose which did not contain immunoreactive protein The results were analysed on a Shimadzu FDU-3 central processing unit enabling quantitation of the immunoblots.

3. RESULTS

We have previously demonstrated that sustained carbachol treatment of HM1-expressing CHO cells results in a reduction in membrane-associated levels of some combination of $G_q\alpha$ and $G_{11}\alpha$ [12]. These two phosphoinositidase C-linked G-proteins comigrate in SDS-PAGE (10% (w/v) acrylamide) and as such it has previously not been possible to assess directly in a single experiment whether both of these G-proteins are regulated by the receptor.

HM1 expressing CHO cells co-express $G_q\alpha$ and $G_{11}\alpha$ (Fig. 1a,b, lanes 1 and 2). Following resolution in the 13% acrylamide SDS-PAGE system described by Blank et al. [14] (Fig. 1a) immunoblotting with an antiserum (CQ3), which was generated against a synthetic peptide which is conserved in the primary sequences of these two G-proteins, demonstrated separation of a purified mixture of these G-proteins (Fig. 1a, lane 3). HM1-expressing CHO cells expressed CQ3 immunoreactive polypeptides which comigrated with each of the purified proteins from liver. In this system $G_q\alpha$ migrates more rapidly than $G_{11}\alpha$ [14]. Resolution of membranes of HM1 expressing CHO cells in 12.5% acrylamide, 0.0625% bisacrylamide (w/v) gels which contained a linear gradient of 4–8 M urea also resolved two CQ3 reactive polypeptides (Fig. 1b) but in this system $G_{11}\alpha$ migrates more rapidly than $G_q\alpha$ [15]. As antiserum CQ3 interacts equally with these two G-proteins, the higher immunoreactivity associated with $G_{11}\alpha$ in both gel systems (Fig. 1a,b) indicates that this polypeptide is more highly expressed in CHO cells is than $G_q\alpha$. Analysis of a number of immunoblots of membranes of HM1-expressing CHO cells following resolution either by the method of Blank et al. [14] or in the 12.5% acrylamide, 0.0625% bisacrylamide (w/v) gels which contained a linear gradient of 4–8 M urea demonstrated that the relative steady-state levels of expression of $G_{11}\alpha$:$G_q\alpha$ were some 2.4:1.0.

Treatment of HM1 CHO cells with the cholinergic agonist carbachol (10^{-3} M, 16 h) reduced membrane-associated immunoreactivity corresponding to both $G_{11}\alpha$ and to $G_q\alpha$ (Fig. 1a,b). Treatment of the cells with varying concentrations of carbachol demonstrated that half-maximal loss of both $G_q\alpha$ and $G_{11}\alpha$ was achieved

Fig. 1. HM1 expressing CHO cells co-express $G_q\alpha$ and $G_{11}\alpha$ and both are down-regulated by treatment with carbachol. (a) Membranes (25 μg) from HM1 expressing CHO cells which were untreated (1), or treated with carbachol (10^{-3} M, 16 h) (2), were resolved in the SDS-PAGE system of Blank et al. [14] along with 10 ng of a purified mixture of bovine liver G_q and G_{11} [14] containing approximately equal amounts of these two proteins (3). (b) Membranes (25 μg) from HM1-expressing CHO cells which were untreated (1), or were treated with carbachol (10^{-3} M, 16 h) (2), were resolved in the SDS-PAGE system urea gradient gel system described in section 2 In both cases the gels were immunoblotted using antiserum CQ3 as primary reagent.

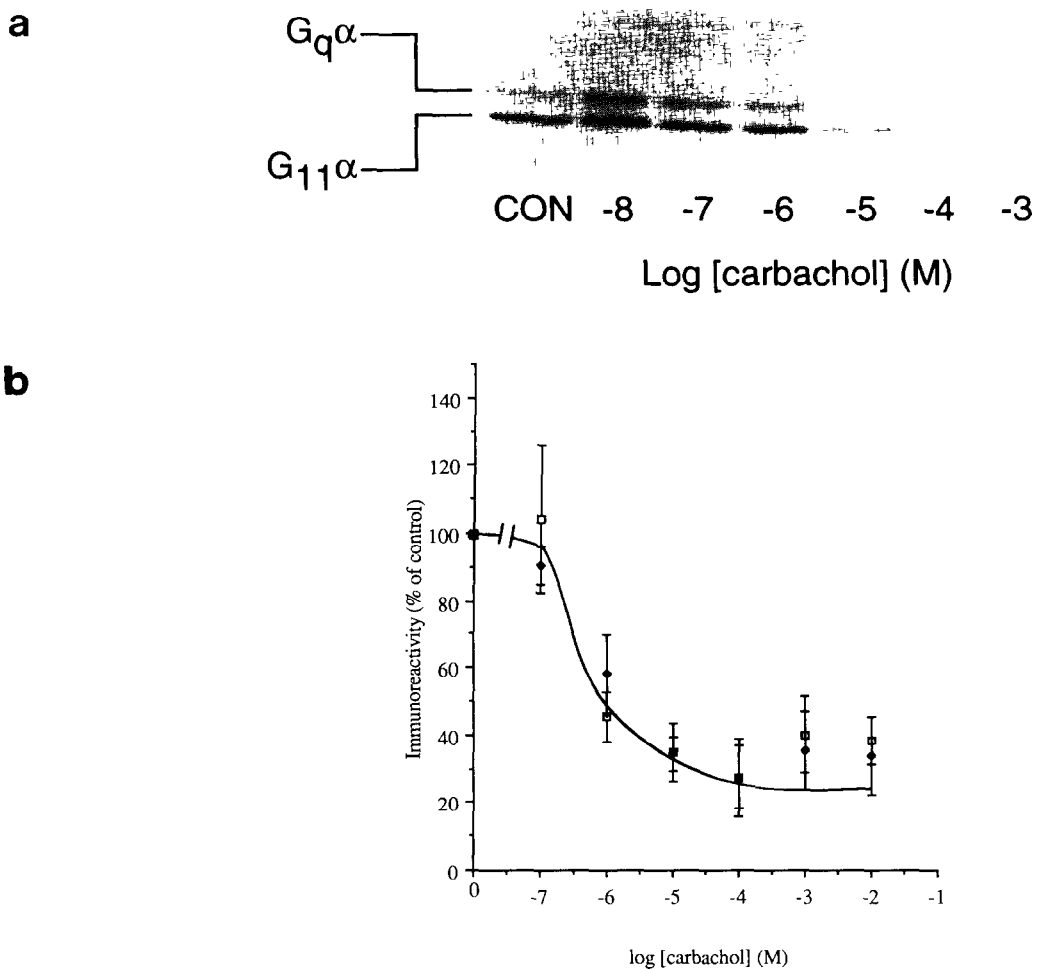

Fig. 2. Exposure of HM1 expressing CHO cells to varying concentrations of carbachol results in equivalent down-regulation of $G_{11}\alpha$ and $G_q\alpha$. HM1 expressing CHO cells were treated with varying concentrations of carbachol for 16 h. Membranes (25 μg) prepared from these cells were resolved by the urea gradient system of [15] and immunoblotted using antiserum CQ3 as primary reagent. (a) Representative example of an immunoblot from such experiments in which the carbachol concentration was varied from 0 (CON) to 10^{-3} M. (b) Composite data of experiments derived from cells from individual treatments. Results are displayed as mean ± S.E.M. (n = 4) of the immunoreactivity recorded from membranes of cells maintained in the absence of carbachol. $G_q\alpha$ immunoreactivity (open squares), $G_{11}\alpha$ immunoreactivity (filled diamonds).

by 16 h treatment with some 10^{-6} M carbachol (Fig. 2). The time-course of down-regulation of $G_q\alpha$ and $G_{11}\alpha$ in HM1-expressing CHO cells exposed to carbachol (10^{-3} M) was also shown to be equivalent (Fig. 3) with half-maximal effects achieved by 4 h. Equivalent data for $G_q\alpha$ loss, both in terms of time-courses and dose–response to carbachol was obtained from experiments in which immunoblots were probed with the $G_q\alpha$-specific antiserum IQB (data not shown).

4. DISCUSSION

Expression of the human muscarinic M1 acetylcholine receptor in CHO cells has been noted to allow cholinergic agonist-dependent down-regulation of the α subunit of a member or members of the G_q family of G-proteins [12]. This effect is dependent upon the time of exposure and the concentration of agonist used [12] and mechanistically has been demonstrated to result from agonist-enhanced proteolysis of the G-protein(s) [13]. It is also specific for G_q/G_{11} as cellular levels of other G-proteins are not modified by such treatment and is dependent on the receptor as carbachol treatment of parental CHO cells does not regulate G_q/G_{11} levels [12].

G_q and G_{11} are the best studied and most widely expressed of the G-proteins (G_q, G_{11}, G_{14} and G_{16}) which comprise the G_q family of G-proteins [5,8]. A series of transfection experiments have shown that the α subunits of the various members of this family allow communication between agonist-occupied receptors and, at least, the β1 isoform of the phospholipase C family of effector enzymes [6,7] resulting in the hydrolysis of phosphatidylinositol-4,5-bisphosphate to generate the second messengers inositol-1,4,5-trisphosphate and 1,2-diacylglycerol.

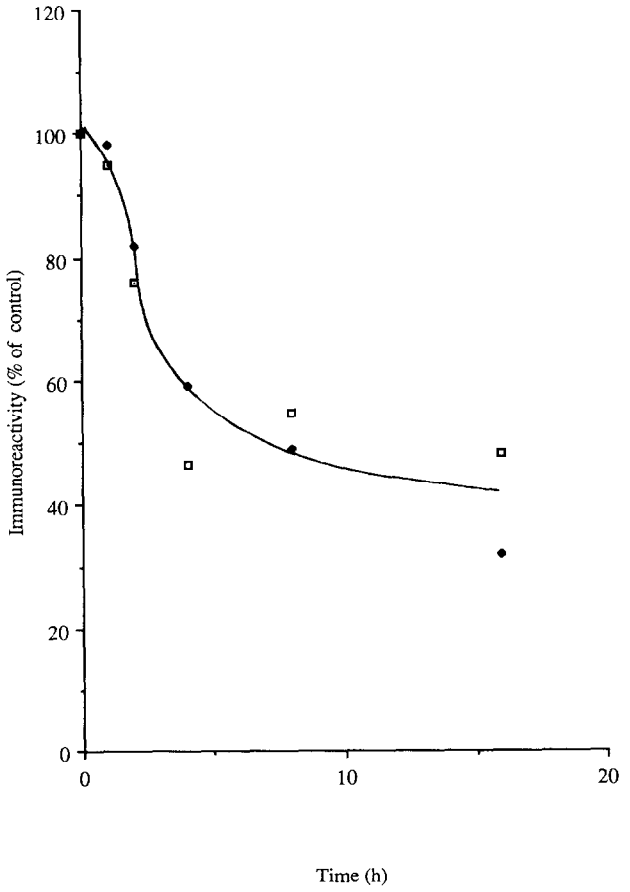

Fig. 3 Exposure of HM1-expressing CHO cells to carbachol for varying times results in equivalent down-regulation of both $G_{11}\alpha$ and $G_q\alpha$. HM1-expressing CHO cells were treated with carbachol (10^{-3} M) for times up to 16 h. Membranes (25 μg) prepared from these cells were resolved by the urea gradient system of [15] and immunoblotted using antiserum CQ3 as primary reagent. A representative example of an immunoblot from such experiments is displayed. $G_{11}\alpha$ immunoreactivity (filled diamonds), $G_q\alpha$ immunoreactivity (open squares). Seven similar experiments were performed on membranes derived from individual treatments of the cells with carbachol.

To date, it has been impossible to assess how selectively, if at all, a receptor is able to interact with different members of the G_q family when they are expressed in the same cell and thus are potentially both available to the receptor. Analysis of mRNA by reverse transcriptase-polymerase chain reaction has shown $G_q\alpha$ and $G_{11}\alpha$ to be co-expressed in these HM1-expressing CHO cells [13] and that agonist treatment does not alter levels of mRNA encoding either of these G-protein α subunits [13]. In a number of systems selective down-regulation of the G-proteins which are activated by receptors has been observed during chronic exposure on the cells to receptor agonists, while cellular levels of other G-proteins with which the receptor does not interact are unchanged [11].

In this study we have used this strategy to examine the relative in vivo interactions of the HM1 receptor with $G_q\alpha$ and with $G_{11}\alpha$. These two G-proteins are some 90% identical in primary sequence [8] and are of extremely similar molecular mass. As such they migrate together in many SDS-PAGE systems. We wished to use an antiserum which would identify both G-proteins equally [18] so that their relative levels in the cell could be observed but this required the development of SDS-PAGE conditions suitable for resolution of the two polypeptides. In this study we have used two such systems. In the 13% acrylamide system described by Blank et al. [14] $G_q\alpha$ migrated more rapidly through the gel than $G_{11}\alpha$ (Fig. 1a). In contrast, in low bisacrylamide gels containing a gradient of urea, the reverse order of mobility was observed [15].

Immunoblotting membranes of HM1 expressing CHO cells with antiserum (CQ3), which was generated against a peptide which represents the C-terminal decapeptide which is completely conserved between the α subunits of these two G-proteins [18], demonstrated that these cells express some 2.5-fold higher steady-state levels of $G_{11}\alpha$ than of $G_q\alpha$. Treatment of the cells with carbachol resulted in a down-regulation of both G_q and G_{11} without altering levels of any other G-protein (data not shown but see [12]). Both varying the concentration of carbachol and the time of exposure to the agonist resulted in down-regulation of the same proportion of each of G_q and G_{11} at each point examined. These results clearly indicate that the HM1 receptor, at least when expressed in CHO cells, does not actively select between $G_q\alpha$ and $G_{11}\alpha$ and interacts with each equally based only on their relative cellular levels. G_q and G_{11} are widely co-expressed (Milligan, G., Mullaney, I. and McCallum, J.F., submitted for publication) and this raises obvious questions as to the specific role of each. To date the only reported difference between them has been in the regulation of TRH receptor function when G_q or G_{11} was co-expressed with this receptor in *Xenopus* oocytes [19]. Further examination of the individual functions of these G-proteins is clearly required.

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