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# Cholera toxin mediated regulation of the expression of Gqα and G11α GTP binding proteins

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Abbreviations: CT, choelra toxin; cAMP, cyclic adenosine monophosphate; db-cAMP, dibutyryl cAMP; FSK, forskolin; PKA, protein kinase A; $IP_3$, inositol 1,4,5-triphosphate; DAG, diacylglycerol

## Abstract

**Previously it has been shown that persistent activation of the stimulatory adenylyl cyclase pathway with cholera toxin (CT) downregulates the Gsα polypeptide (80%) in a cAMP-independent manner in C6 glioma cells (Shah, 1997). This study was conducted to examine the short and long term effects of CT on the regulation of pertussis toxin-sensitive and -insensitive G proteins and their transcripts in C6 glioma cells. Treatment of C6 cells with CT (100 ng/ml) up to 16 h had no effect on either Gi or Gq/11α proteins. However, prolonged exposure (24-48 h) caused increased expression of Gi (20-30%) and Gq/11α proteins (40%). Urea gradient gels, which can separate Gqα and G11α proteins, revealed that prolonged CT treatment increased the expression of both of these G proteins. The CT-mediated enhanced expression of Gqα and G11α proteins was accompanied by increased mRNA levels of these proteins as determined by RT/PCR. Cyclic-AMP elevating agents like forskolin (10 μM) and db-cAMP (1 mM) mimicked the effect of CT on Gi but not Gq/11α proteins. These studies show long term cAMP-dependent regulation of Gi and cAMP-independent expression of Gq/11α proteins in C6 glioma cells.**

**Keywords:** G-proteins, cholera toxin, C6 glioma cells, forskolin, dibutyryl cAMP, protein kinase A.

## Introduction

The heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins) mediate a variety of cellular processes by coupling transmembrane receptors to different effector molecules, including adenylyl cyclase and inositol phospholipid-specific phospholipase C (PLC) (Exton, 1997; Eyster, 1998). Activation of Gs/adenylyl cyclase results in the production of cyclic AMP (cAMP) and activation of cAMP-dependent protein kinase (PKA). Activation of Gi protein leads to inhibition of adenylyl cyclase activity and thus decrease in cAMP levels (Sunahara *et al*., 1996). The pertussis toxin-insensitive G-proteins include a family of Gq proteins (Gq, G11, G15 and G16) which mediate their effects by activating PLC and thus generating second messengers, inositol-1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG), thereby leading to activation of protein kinase C (PKC) and the mobilization of intracellular calcium, respectively (Berridge, 1993, Smrcka *et al*., 1991). The two members of the Gq family, Gq and G11, are widely and quite often co-expressed in the body tissues (Milligan, 1993a).

Our previous studies have shown that rat C6 glioma cells express all these G-proteins (Gs, Gi, Gq and G11) (Shah and Milligan, 1995). C6 glioma cells are a bipotential cell line with oligodendrocytic and astrocytic properties. C6 cells can differentiate into type 2 astrocytes by addition of agents that increase intracellular cAMP (Messens and Slegers, 1992). These cells endogenoulsy express receptors for a number of neurotransmitters including the adrenergic system and have proved to be a valuable model for studying the G-protein coupled signal trans-duction cascade (Hosoda *et al*., 1994; Kato *et al*., 1996). Cyclic AMP has been shown to exert multiple effects in C6 cells. These include differentiation of these cells towards more astrocyte phenotype (Segovia *et al.*, 1994), regulation of adrenergic receptors (Hosoda *et al.*, 1994), induction of interleukines (Slegers and Joniau, 1996) heat shock proteins (kato *et al*., 1996) and prostaglandin synthesis (Peterson *et al.*, 1996). It has been shown that PLC activation and phosphoinositide breakdown can be modulated by the adenylyl cyclase pathway and this effect is mediated by PKA (Hadcock *et al.*, 1990; Liu and Simon, 1996). It has been shown that persistent CT-treatment of C6 glioma cells causes marked down-regulation of stimulatory G protein, Gsα (Shah, 1997), however, little is known about the regulation of Gi and Gq/11α proteins. The present study was carried out to examine the effects of short and long term exposure of C6 cells to CT and agents that increase intracellular cAMP (forskolin and dibutyryl cAMP) on the expression of Gi and Gq/G11 proteins and their mRNA levels.

## Materials and Methods

### Materials

Taq polymerase and PCR buffer were from Promega. The cDNA synthesis kit was from Pharmacia LKB. Cholera toxin and all other chemicals were reagent grade and purchased from Sigma Chemical Co. All materials for tissue culture were from Gibco/BRL. Antibodies to various G-proteins were kindly provided by Dr. Graeme Milligan (University of Glasgow, UK).

### Cell culture

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) in 5% $CO_2$ at 37˚C. Cells were grown in 75 $cm^3$ tissue-culture flasks and were harvested just before confluency. For these experiments, cells up to 25 passages were used. The treatment of cells with cholera toxin (100 ng/ml), dB-cAMP (1 mM) or forskolin (10 µM) for different times was carried out as described in the figure legends.

### Preparation of membranes

Membranes were prepared from the cells by homogenization with a Teflon-on-glass homogenizer and differential centrifugation as previously described (Shah and Milligan, 1994). Frozen cell pellets were suspended in 5 ml of 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5 (buffer A) and the cells ruptured with 25 strokes of 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 supernatent was further centrifuged at 48,000 *g* for 10 min. The pellet from the second centrifugation was washed with buffer A and recentrifuged at 48,000 *g* for 10 min. Finally the pellet was resuspended in buffer A to yield a protein concentration of 1-3 mg/ml and stored at -80˚C until required. Protein concentration was measured according to the method of Lowry et al (1951).

### Western blots

The generation and specifities of the various antisera used in this study are already described (Mitchell *et al.*, 1991). Membrane samples were resolved by SDS/PAGE in 10% (w/v) acrylamide gels overnight at 60 V. For resolving Gqα and G11α, urea linear gradient gels were prepared and run overnight at 100 V. Proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 3 h in 5% (w/v) gelatin in phosphate buffered saline (PBS), pH 7.5. Primary antisera were added in 1% gelatin in PBS containing 0.2% Nonidet P-40 (NP-40) and incubated overnight. The primary antiserum was removed and blots washed extensively with PBS containing 0.2% NP-40. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase; HRP) in 1 % gelatin/PBS/0.2% NP-40 was added and left for 3h. After removal of the second antiserum, blots were washed extensively as above and developed with o-dianisidine hydrochloride as substrate for HRP. The developed immunoblots were scanned and quantified as described previously (Shah *et al.*, 1995)

### Reverse transcription/polymerase chain reaction (RT/PCR)

Total RNA was extracted according to the acid phenol/guanidinium thiocyanate method of Chomcynski and Sacchi (1987) using RNAzole B. Purity and quantification of RNA were assessed by the ratio of absorbance at 260 and 280 nm. The RT/PCR procedure was carried out as described previously (Shah *et al.*, 1995b). Briefly, samples of 5 mg RNA (20 ml) were denatured by incubation at 65˚C for 10 min followed by chilling on ice and reverse transcribed in 33 ml of reaction mixture using the first strand cDNA synthesis kit (Pharmacia LKB Biotechnology) as detailed by the manufacturer. Incubation was carried out at 37˚C for 1 h and the reaction was stopped by heating samples at 95˚C for 5 min followed by chilling on ice. PCR reactions were carried out using the following 24-mer primers for the a-subunit of the G-proteins.

Gsα-sense, 5' CCACCTGAATTCGAGCATGCC 3'; Gsα-antisense, 5' GCGTGGGTCCTCT CCGGGCTCGGG 3';
Gqα-sense, 5'ATGACTTGGACCGTGTAGCCGACC 3'
G11α-sense, 5' ACGTGGACCGCATCGCCACAGTAG 3';
Gq/11α-antisense, 5' CCATGC GGTTCTCATTGTCTGACT 3', HPRT-sense, 5' CCTGCTGGATTACATTAAAGCACT 3' and HPRT-antisense, 5' CCTGAAGTACTCATTATAGTCAAG 3'.

Amplifications were performed in 100 ml of buffer containing 20-40 pmol of primers, 2.5 units of Taq polymerase in a HYBAID Omnigene temperature cycler. Cycles were as follows: 95˚C/5 min, 60˚C/30 s, 72˚C/1 min (1 cycle); 95˚C/30 s, 60˚C/30 s, 72˚C/1 min (25-30 cycles); 95˚C/30 s, 60˚C/30 s, 72˚C/5 min (1 cycle). These conditions ensured a linear relationship between the amount of template mRNA and the amount of amplified product (Shah *et al.*, 1995). Either the constitutively expressed housekeeping *hprt* gene (Steel and Buckley, 1993) or β-actin (Mullaney *et al*., 1995) was selected as a reference tempelate for this purpose. Reaction products were separated by 1.5-1.75% agarose gel electrophoresis. Data analysis was performed using the Kaleidagraph (version 2.1) curve fitting package driven by an Apple Macintosh computer.

## Results and Discussion

The membranes prepared from C6 cells exposed to CT (100 ng/ml) and other agents for different time periods were subjected to immunoblotting with antipeptide antiserum which was generated against the a-subunit of Gi and Gq/11 proteins. CT-treatment (100 ng/ml) of C6 cells for 24 or 48 h caused little increase (25 ± 6%; mean

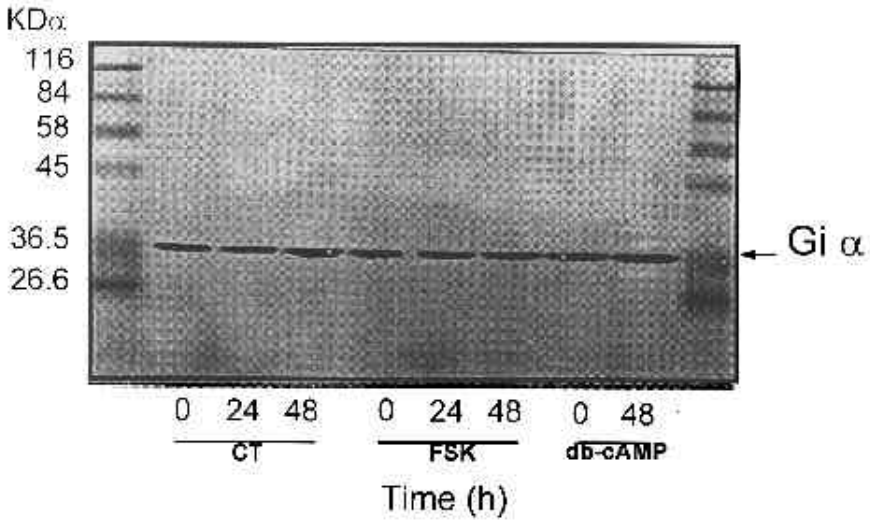


**Figure 1.** Effect of cholera toxin (CT; 100 ng/ml), forskolin (FSK; 10 μM) and dibutyryl cAMP (db-cAMP; 1 mM) on immunoreactivity of membrane Gi2α levels in C6 cells at various time periods. Immunoblots of a representative experiment are shown here (n=4).

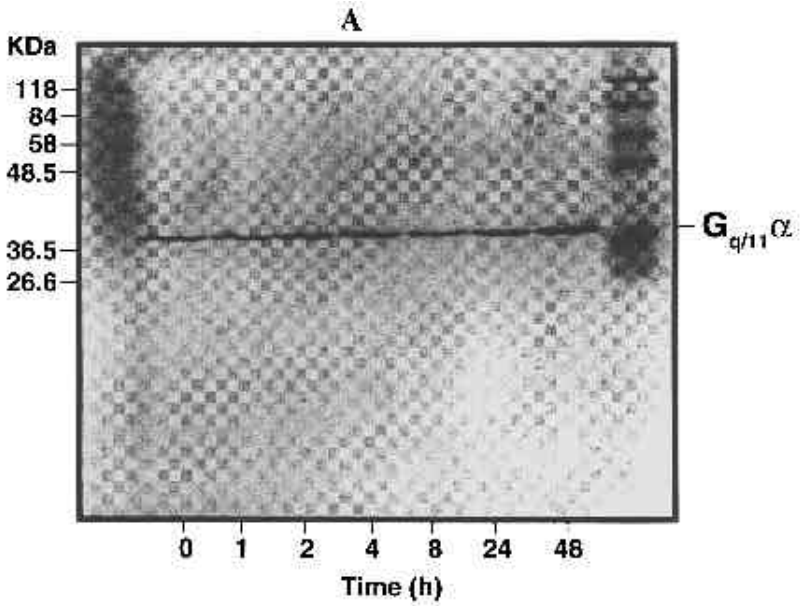


**Figure 2.** (A). Immunological detection of a combination of Gq/G11α proteins at various time points of CT treatment (100 ng/ml). Membranes prepared from C6 cells were resolved by SDS-PAGE (10% w/v, acrylamide) and immunoblotted using antiserum CQ2 (B). Quantitation of data from Figure 4A (Mean ± SD, n=4).

and SEM) in the membrane Gi2α levels (Figure 1). However, CT (100 ng/ml) treatment for 24 and 48 h increased levels of Gq/11α (40%) as detected by immunoblotting using antiserum which was generated against a region that is completely conserved between the two proteins, Gqα and G11α (Mitchell *et al*., 1991) as shown in Figure 2.

Since CT-mediated ribosylation of Gsα results in marked production of cAMP within the cells (Chang and Bourn, 1989), it was tested if the increased intracellular cAMP levels were responsible for effects of CT on G proteins. For that, cells were treated with agents that increase intracellular cAMP like forskolin (FSK) or a cell permeable cAMP analogue (db-cAMP). Treatment of C6 cells with either FSK (10 μM) or db-cAMP (1 mM) for 48 h caused little increase (25 ± 5%) in the level of the Gi2α (Figure 1). The effects of CT on Gq/11α were not mimicked by treatment with both FSK and db-cAMP (data not shown).

To examine the effect of CT on individual polypeptides (Gqα and G11α), urea containing SDS-PAGE was used to resolve Gqα and G11α as described previously (Shah and Milligan, 1994). Quantitation of the CT-induced changes in Gqα and G11α demonstrated that a similar percentage of each G protein polypeptide was regulated at different time periods (Figure 3). To see whether the changes produced in G proteins are due to alterations in their transcripts, the mRNA levels were measured by RT/PCR (Shah et al., 1995). Results show that CT enhanced the expression of G11α and

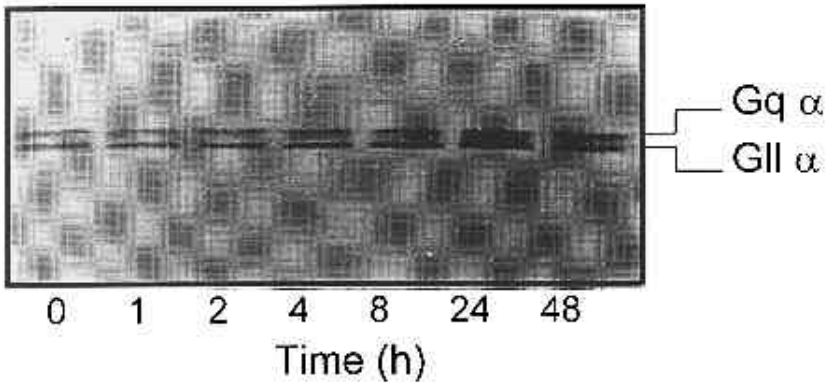


**Figure 3.** Time-course effect of CT (100 ng/ml) on Gqα and G11α proteins. Membranes were resolved by SDS-PAGE (12.5% acrylamide/0.0625% bisacrylamide) with a linear gradient of 4-8 M urea and were immunoblotted with antiserum which recognizes both G11α and Gqα. Under these conditions G11α migrates more rapidly than Gqα (Shah *et al.*, 1994).

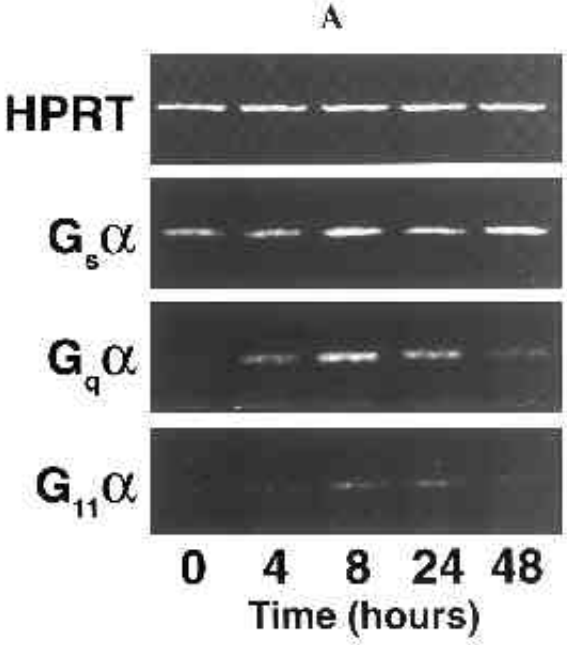


**Figure 4.** (A): RT/PCR analysis of HPRT (as control), Gsα, Gqα and G11α mRNA levels in C6 cells treated with CT (100 ng/ml) for various time periods. (B): The RT/PCR data is quantitated by normalization to HPRT mRNA as an internal standard (Mean ± SD, n=3).

Gqα (60-75%) transcripts without any appreciable change in Gsα mRNA (Figure 4).

CT-induced increase in cAMP occurs as a result of ADP-ribosylation of the a-subunit of stimulatory G-protein (Gs) and thus constitutive activation of adenylyl cyclase (Chang and Bourn, 1989). While CT markedly reduces the membrane Gsα levels, it increases Gi2α and Gq/11α levels at 24-48 h of treatment in C6 cells. The downregulation of Gsα was not produced by increased cAMP levels as the effect could not be reproduced by forskolin or cAMP analogue, db-cAMP (Shah, 1997). It has been suggested that ADP-ribosylation of Gsα increases the protein's susceptibility to proteolytic degradation (Levis and Bourn, 1992; Wedegaertner and Bourn, 1994), mainly through depalmitoylation which causes loosening of Gsα attachement from the cell membrane (Levis and Bourn, 1992, see Mumby, 1997). Such a reduction in the level of G-protein is, in fact, an adaptive mechanism to protect cells from the excessive stimulation of the signalling cascade (Milligan, 1993b). Similar responses are also observed with other G-proteins like Gqα and G11α upon prolonged receptor stimulation with pharmacological doses of the receptor agonist (Shah and Milligan, 1994; Shah *et al.*, 1995).

Measurment of mRNA levels by RT/PCR showed that the ability of CT to produce a reduction in levels of membrane Gsα was not related to alterations in levels of Gsα mRNA. This is consistent with the observations of Carr *et al.* (1990) but in contrast to findings of Lin *et al.* (1993) in GH3 lactotrophs where the later authors showed a biphasic response to CT; an initial increase and then decline in both Gsα mRNA and protein. Our previous studies in NCB20 cells transfected with β2-adrenoceptor showed that isoproterenol mediated decrease in Gsα protein was independent of any changes in its transcript (Mullaney *et al.*, 1995). It seems as reduction in membrane Gsα requires either occupancy of receptors with the agonists or needs a direct action (e.g., ADP-ribosylation) on the Gsα protein (Levis and Bourn, 1992).

In C6 cells, the upregulation of Gq/11α proteins in response to CT (100 ng/ml) was observed only after prolonged exposure. It has been shown that PLC activation and phosphoinositide breakdown can be modulated by the adenylyl cyclase pathway and this effect is mediated by PKA (Hadcock *et al.*, 1990; Liu and Simon, 1996). Moreover, cAMP-mediated transcriptional regulation of G-proteins (Hosoda *et al.*, 1994; Mckenzie and Milligan, 1990) and the linked receptors (Collins *et al.*, 1989; Thomas *et al.*, 1992) is well documented. Recent studies have shown that cAMP-mediated regulation of adrenergic receptors occur at the level of gene transcription, not mRNA stability (Hosoda *et al.*, 1994). Further

studies in this direction in C6 glioma cells are required.

cAMP exerts multiple effects on various enzymes and thus alters cellular activities. Such effects are tissue-dependent. For example cAMP causes induction of interleukine-6 in C6 cells which corresponds well with the differentiation of these cells (Slegers and Joniau, 1996). Cyclic AMP causes differentiation of C6 cells towards more astrocyte phenotype as reflected by a decrease in neuronal marker glutamate decarboxylse and increased expression of glial fibrillary acidic protein (Messens and Slegers, 1992). Present studies show that upregulation of Gqα and G11α proteins by CT occurs only after prolonged exposure (24 and 48 h) and this effect seems to be specific to C6 cells as it was not seen in other cells like pituitary gonadotrophs (unpublished results). Similarly induction of heat shock proteins by cAMP occurs only in C6 glioma cells but not in other neuronal cell lines (Kato *et al.*, 1996). Thus, the possibility that regulation of PLC-coupled G proteins (Gq/11α) through long term stimulation of the adenylyl cyclase pathway is a result of differentiation of C6 cells can not be overlooked.

While exposure to CT downregulated Gsα, it led to upregulation of Gq/11α and Gi2α proteins with no significant changes in Go or G-protein β-subunits. Earlier studies in C6 cells (Carr *et al.*, 1990) and NG-108 cells (Mckenzie and Milligan, 1990) show no change in Gia protein in response to increase in intracellular cAMP. Contrary to that forskolin and isoproterenol increase the expression of Gi2α protein in S49 cells (Hadcock *et al.*, 1990). Mullaney *et al*. (1988) reported that prolonged treatment for 8 days of neuroblastoma x glioma hybrid cells with dibutyryl cAMP increased the expression of Goa but decreased Gia proteins. Taken together our previous and present studies, it is evident that CT exhibits diverse effects on G-proteins (Gsα, Gi2α and Gq/11α) and shows long-term cross regulation between stimulatory Gs/adenylyl cyclase cascade and phosphoinositidase C coupled Gq/11α proteins.

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