Cholera Toxin Mediated Cross Regulation between Stimulatory G Protein and Interleukin-1 in Mouse Intestine

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Summary: Cholera toxin (CTX) is produced by virulent strain of *Vibrio cholerae*. CTX binding to the α-subunit of G-proteins causes its ADP-ribosylation and thus constitutive activation of the Gs/adenylyl cyclase pathway leading to increased intracellular cyclic AMP levels. cAMP can modulate the expression of various genes including interleukin-1 (IL-1). This study was undertaken to examine the CTX-mediated cross regulation between the regulation of G-proteins and IL-1. Isolated pieces of intestine (40 mg) from neonatal mice were kept in RPMI medium supplemented with 10% fetal calf serum and treated with CTX (1 μg/ml) for various time periods until 4 h. The G protein and IL-1α levels were measured by Western blots and ELISA respectively. The results show that CTX caused marked reduction in the Gsα protein with out any effect on Gi or Go proteins. CTX treatment also caused 2-3 fold increase in the production of IL-1α levels compared to control untreated tissues and the effects were mimicked by treatment with forskolin (a direct activator of adenylyl cyclase) and dibutyryl-cAMP (a cAMP analogue) indicating that CTX-mediated effects on IL-1α were cAMP-dependent. Pretreatment with the nitric oxide (NO) synthase inhibitor, L-NAME (10 μM), decreased the levels of IL-1α (60%) compared to control. Similarly pretreatment of tissues with cyclooxygenase inhibitor (indomethacin; 10 μM) blocked an increase in IL-1α levels. It seems that the CTX-mediated regulation of IL-1α is cAMP-dependent and involves multiple signalling pathways, i.e., the activation of the NO and COX pathways in mouse intestines.

Introduction

Cholera toxin (CTX) is the *Vibrio cholera* enterotoxin that exerts its effects on the small intestine *in vivo* through activation of guanine nucleotide binding protein (G protein). The event is triggered by the A subunit of cholera toxin, resulting in increased cAMP levels that eventually causes diarrhoea and fluid loss that is characteristic in patients with cholera [1,2]. CTX-mediated increase in intracellular cAMP occurs as a result of activation of Gs/adenylyl cyclase. On the other hand, activation of Gi protein leads to inhibition of adenylyl cyclase activity and thus decrease in cAMP levels [3,4]. Cyclic AMP has been shown to exert multiple effects in cells. These include differentiation of cells [5], regulation of adrenergic receptors [6] and induction of cytokines [7-9]. We have recently shown that cAMP increases the expression of Gq/11 proteins [10] that are coupled with activation of phospholipase C and generation of second messengers inositol tri-phosphate (IP_3) and diacylglycerol (DAG).

IL-1 is a low molecular weight (17 kDa) immunoregulatory protein produced by stimulated macrophages as well as other cell types. IL-1 affects nearly every cell type and often exerts its effects in concert with other cytokines or small molecule mediators. Though IL-1 is considered highly inflammatory cytokine and the margin between clinical and unacceptable toxicity in human is exceedingly narrow [10], there is much experimental data illustrating a role for IL-1 in boosting natural host defense mechanisms. In fact small amounts of IL-1 production in disease are necessary for maintaining natural host defences whereas high amounts of IL-1 are lethal [11]. *In vitro* tissue culture studies have revealed that IL-1 causes rapid hydrolysis of GTP with or without an increase in adenylyl cyclase activity, hydrolysis of phospholipids and release of arachidonic acid. In addition, IL-1 mediated tyrosine phosphorylation have been reported [10,11].

Though extensive studies have been carried out on unravelling the factors associated with induction of cytokines in various pathological conditions, very little is known about the CTX-mediated cross regulation between activation of G

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proteins and cytokine induction. Moreover, the exact mechanism of CTX-mediated regulation of cytokines is not fully understood. The present study was carried out to examine the effects of CTX and agents that increase intracellular cAMP (forskolin and dibutyryl cAMP) on the expression of G proteins and IL-1.

Results and Discussion

The membranes prepared from tissues exposed to CTX (1 μg/ml) and other agents for different time periods were subjected to immunoblotting with antipeptide antiserum which was generated against extreme carboxy terminus of α-subunit of various G proteins (Table 1). CTX treatment rapidly decreased the long form (51 kDa) of Gsα-protein. CT-mediated decrease in Gsα immunoreactivity was rapid (40%) and could be observed at 4 h of treatment (Fig. 1). Under similar conditions, CTX did not affect the expression of inhibitory G protein Gi or Go (data not shown).

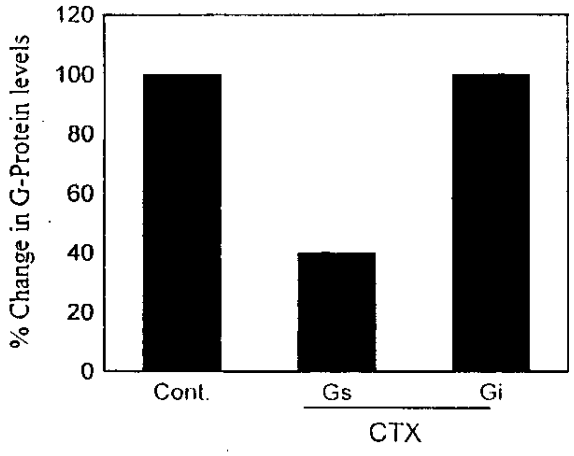

Fig. 1: Effect of cholera toxin (CTX) on the immunoreactivity of stimulatory (Gsα) and inhibitory (Giα) G proteins in intestinal tissue *in vitro*. The isolated pieces of ileum kept in RPMI medium supplemented with 10% fetal calf serum were treated with CTX (1 μg/ml) for 4 h, membranes prepared and Western blotting done as detailed in Methods. CTX downregulates the Gsα protein without any effect on Giα protein.

Since CT-mediated ribosylation of Gsα results in marked production of cAMP within the cells [3,16], we tested if the increased intracellular cAMP levels are responsible for effects of CT on G proteins and cytokines. For that intestinal tissues were treated with agents that increase intracellular cAMP; like forskolin (FSK) or cAMP analogue (db-cAMP). Treatment of intestinal tissue either with FSK (10 μM) or db-cAMP (1 mM) did not decrease the levels of either form of Gsα indicating cAMP-independent regulation of Gsα by CTX (Fig.1). However, CTX (1 μg/ml) treatment of intestinal tissue *in vitro* caused 2.5-3 fold increase of IL-1α levels as determined by ELISA. This effect of CTX was mimicked by cAMP-elevating agent (forskolin; 10 μM) or permeable cAMP analogue (dB-cAMP 1mM) as shown in Fig. 2.

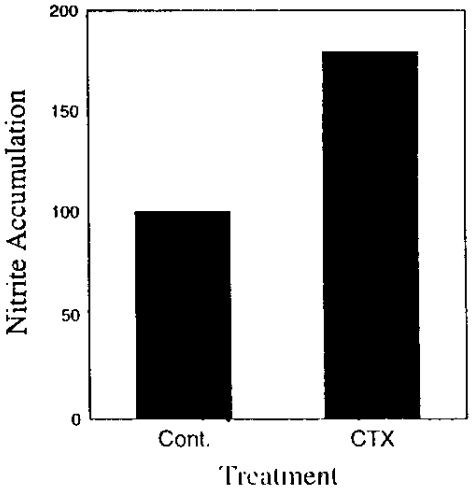

Fig. 2: Cholera toxin (CTX), forskolin (FSK; 10 μM) and dibutyryl cAMP (dB-cAMP; 1 mM) increase the production of interleukine-1a (IL-1a) in intestines. The isolated pieces of mice intestine were treated with CTX (1 μg/ml) as described in Fig.1. The IL-1α released into the medium was measured by ELISA as described in Methods.

Since nitric oxide (NO) is reported to be involved in the induction of IL-1, we tested if NO production under the effect of CTX has any role in IL-1 induction. Results in Fig. 3 show that CTX treatment (1 μg/ml) stimulated the production of NO in intestinal tissue. Pretreatment of tissues with NO synthase (NOS) inhibitor, N-nitro-L-arginine methyl ester (L-NAME; 20 μM) inhibited CTX-induced

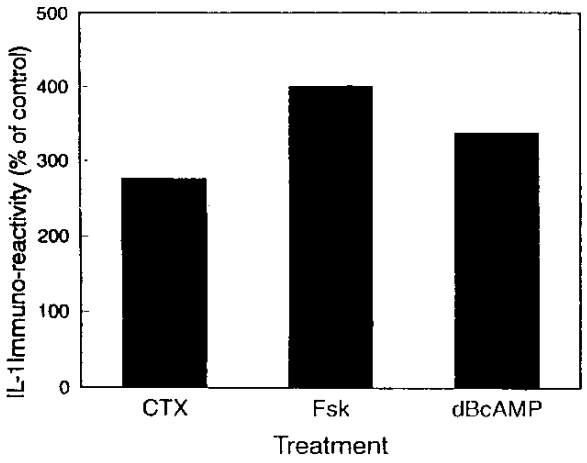

Fig.3: Effect of cholera toxin (CTX) on the induction of nitric oxide (NO) in intestinal tissue. The intestines were treated with CTX (1 μg/ml) and NO was measured using Griess method.

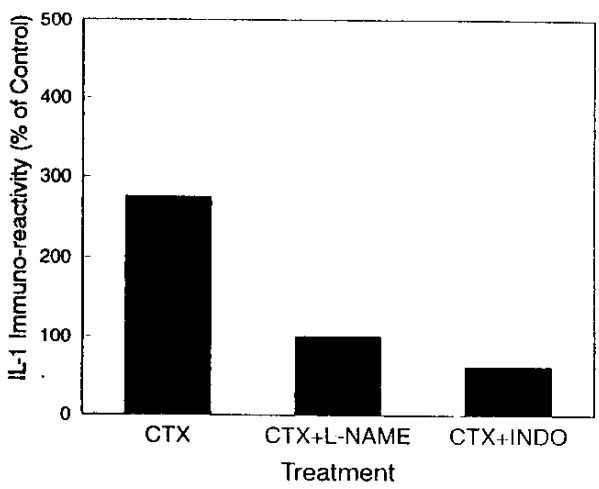

Fig. 4: Effect of nitric oxide synthase (NOS) inhibitor, L-NAME (20 μM) and cyclooxygenase inhibitor, indomethacin (10 μM) on the expression of IL-1α in mice intestine. L-NAME and indomethacin were added into the culture medium before addition of CTX. Both L-NAME and indomethacin inhibit the stimulatory effect of CTX on IL-1 production.

production of IL-1α (Fig. 4). Similarly treatment with cyclooxygenase (COX) inhibitor, indomethacin (10 μM), blocked the production of IL-1α in intestinal tissue (Figure 4).

CTX-induced increase in cAMP occurs as a result of ADP-ribosylation of the α-subunit of stimulatory G-protein (Gs) and thus constitutive activation of adenylyl cyclase [3,16]. Present study shows that CTX treatment for 4 h or longer markedly reduces the membrane Gsα levels, however, it increases the expression of IL-1α in isolated tissue from small intestine (ileum) of mice. There was no effect of CTX on Giα, Go proteins or β-subunits of G proteins which is similar to our earlier findings in rat C6 glioma cells [9].

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. It was previously suggested that ADP-ribosylation of Gsα increased the protein's susceptibility to proteolytic degradation [16,17]. Recent studies have shown that activation of Gsα protein either by CT [18,19], constitutive activating mutation or prolonged exposure to neurotransmitter and β-adrenoceptor agonists induce a loss of Gsα from the plasma membrane [17-20]. This process is suggested to occur mainly through depalmitoylation which causes loosening of Gsα attachement from the cell membrane [18]. 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. Similar responses are observed with other G-proteins like Gqα and G11α upon prolonged receptor stimulation with pharmacological doses of the agonist [14]. While exposure to CT downregulated Gsα protein, no significant changes were seen in the levels of pertussis toxin sensitive G-proteins, Gi2α, Go or G-protein β-subunits. This confirms the previous findings in NG-108 cells [21], C6 glioma cells [22] and chick sympathetic neurons [19].

Maintenance of homeostasis during infection and injury requires stimulation of the immune system, elimination of micro-organisms, self-protection of the host against toxic inflammatory processes and tissue repair. The organization of this system is controlled by soluble tissue mediators called cytokines or interleukines. The cytokines

involved in this group include IL-1, IL-6 and Tumor necrosis factor (TNF-a) [10,11, 23,24]. IL-1 production has been reported in patients with various viral, bacterial, fungal and parasitic infections; leukemias, HIV-1 infection; autoimmunity disorders; trauma (surgery); ischaemic diseases, asthma, and transplant rejection [10,11]. Our results show that CTX-mediated stimulation of cells causes marked production of IL-1 which is cAMP-dependent. This was supported by the fact that treatment of tissues with cAMP increasing agents (forskolin and dB-cAMP) caused an increase in the level of IL-1. Thus, a strong relationship between activation of stimulatory G protein (Gsα) and the induction of IL-1 is observed in the mice intestines. Besides CTX, other toxins such as bacterial endotoxin, lipopolysaccharide (LPS), also down regulates the G proteins. The level of Gi3α protein was markedly decreased compared to Gsα and Gi2α proteins in rat peritoneal macrophages [25].

The generation of nitric oxide (NO) in disease appears to be a fundamental event [26]. Previous studies have shown that IL-1 induces NOS [10]. In fact IL-1 mediated induction of NOS is augmented by elevated cAMP levels which accounts for a number of biological effects [26]. Our results show that blockade of NOS by L-NAME resulted in decreased production of IL-1α in response to CTX treatment. Similar findings were reported earlier in mast cells and macrophages treated with lipopolysaccharide (LPS) [27]. It seems as both IL-1α and NO regulate the induction of each other. The cascade is further complicated due to the fact that NO also induces the activation of cyclooxygenase pathway [28].

Treatment of intestinal tissue with cyclooxygenase (COX) inhibitor, indomethacin, resulted in CT-induced decrease in IL-1α levels. This suggest the possible involvement of COX pathway in the induction of IL-1. In fact it has been reported that anti-inflammatory drugs like corticosteroids and COX inhibitors (indomethacin, salicylic acid) reduces the inflammatory response in human beings [29]. In conclusion our studies demonstrate the involvement of multiple signalling pathways in the CTX-mediated induction of IL-1 and also show a cross regulation between G protein and cytokine pathways in mouse intestines.

Experimental

Materials

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). ELISA kits for measuring cytokines were obtained from Amersham, UK.

Cholera toxin treatment

The pieces of small intestine were isolated from mice and washed with Tyrode's solution. The tissue was kept in RPMI medium supplemented with 10% fetal calf serum and treated with CTX (1 μg/ml) for various time periods up to 4 h. For measuring the effect of cAMP, tissues were treated with either cAMP analogue, dB-cAMP (1 mM) or forskolin (10 μM) for different times as described in figure legends.

Preparation of membranes

Membranes were prepared from the tissues by homogenization with a Teflon-on-glass homogenizer and differential centrifugation as described [12]. Frozen tissue pieces were suspended in 5 ml of 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5 (buffer A) and ruptured with 25 strokes of homogenizer. The resulting homogenate was centrifuged at 500 X 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 48000 X g for 10 min. The pellet from the second centrifugation was washed with buffer A and recentrifuged at 48000 X g for 10 min. Finally the pellet was resuspended in buffer A at a protein concentration of 1-3 mg/ml and stored at -80 °C until required. Protein concentration was measured according to the method of Lowery *et al* [13].

Western blots

The generation and specificities of the various antisera used in this study for immunoblotting are already described [9,11,13] and shown in Table 1. Membrane samples were resolved by SDS/PAGE in 10% (w/v) acrylamide gels overnight at 60 V. Proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2-3 h in

Table 1: Specificities and generation of anti-G protein antisera. Each antiserum was generated in a New Zealand White rabbit using a conjugate of the synthetic peptide and keyhole limpet hemocyanin, as described previously (21,22).

Antiserum	Peptide used	G protein sequence	Antiserum identifies
CQ2	QLNLKEYNLV	$G_{q\alpha}$ 351-360 $G_{11\alpha}$ 350-359	$G_{q\alpha}$, $G_{11\alpha}$
SG1	KENLKDCGLF	Transducin a 341-350	Transducin, $G_{i1\alpha}$, $G_{i2\alpha}$
CS1	RMHLRQYELL	$G_{s\alpha}$ 385-394	$G_{s\alpha}$
BN3	MSELDQLRQE	β_1 1-10	β_1, β_2
IQB	EKVSAFENPYVDAIKS	$G_{q\alpha}$ 119-134	$G_{q\alpha}$

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 horesradish peroxidase; HRP) in 1 % gelatin/PBS/0.2% NP-40 was added and left for 3h. After removal of second antiserum, blots were washed extensively as above and developed with o-dianisidine hydrochloride as substrate for HRP as described [11]. The developed immunoblots were scanned and quantified as described [12,14].

Cytokine measurement

The levels of IL-1α were determined using ELISA kits (Amersham Co., UK). The assay system was based on a solid phase ELISA which utilized a monoclonal antibody for mouse IL-1α bound to wells of a microtitre plate together with a biotinylated antibody to (m)IL-1α and streptavidin conjugated to horseradish peroxidase. This assay system was able to measure IL-1α in the range of 15.6-1000 pg/ml (0.78-50 pg/well).

Measurement of Nitric oxide (NO) release

No release was determined by measuring the accumulation of nitrite. Nitrite was measured by a spectrophotometric assay based on the Griess reaction (15). Briefly after an incubation of tissues with CTX (1 μg/ml), the medium of each well was mixed with an equal volume of the Griess reagent (1% sulphanilamide/0.1% naphthylethylenediamine dihydrochlorde/2.5% H_3PO_4) and incubated at room temperature for 10 min to yield chromophore. The absorbance was read at 543 nm, using a Beckman spectrophotometer. Sodium nitrite ($NaNO_2$) was used as standard. The nitrite amount was calculated from a standard curve.

Acknowledgements

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