Correlation between prolactin secretion and G_s protein expression during sustained cholera-toxin stimulation

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We have studied the chronic effect of cholera toxin (CTX) on prolactin synthesis and secretion in GH_3 pituitary-tumour cells. Time-course analysis showed that prolactin secretion increased with time of CTX exposure, reached a peak at 3 h, and decreased thereafter. Prolactin synthesis was also shown to be stimulated by CTX. The basic and forskolin-stimulated cyclic AMP levels of the CTX-treated cells followed a biphasic time response similar to that of prolactin secretion. Exposure of cells to CTX for more than 3 h abolished the subsequent CTX-catalysed ADP-ribosylation *in vitro*. Moreover, a significant decrease in the pertussis-toxin-catalysed ADP-ribosylation was found after cells were

exposed to CTX for longer than 6 h. Western-blot analysis indicated that the amount of $G_s\alpha$ (α -subunit of G_s) protein increased within 3 h, followed by a gradual decrease to 50 % of the control level at 24 h. The accumulation of $G_s\alpha$ mRNA increased within 6 h of CTX exposure, and decreased thereafter to 40 % of the basal level at 48 h. Our findings that prolonged treatment of CTX induced similar patterns of time responses in $G_s\alpha$ protein expression, cyclic AMP production and prolactin secretion indicate that CTX-induced changes in $G_s\alpha$ protein levels may be responsible for the cellular response leading to prolactin secretion.

INTRODUCTION

Prolactin secretion in the pituitary is under the control of a complex array of hormones and neurotransmitters [1–4]. For example, dopamine inhibits [5,6], whereas thyrotropin-releasing hormone (TRH) [7,8] and vasoactive intestinal peptide (VIP) [9,10] stimulate, prolactin secretion. The effects of some of these hormones are mediated through different G-proteins that activate many signal-transduction pathways; thus VIP stimulates G_s protein and increases cyclic AMP production [11,12], whereas TRH was implicated in activation of phosphoinositide turnover through G_q/G_{11} protein [13–15]. GH_3 rat pituitary-tumour cells [16] co-express both prolactin and growth hormone. Except for the lack of responsiveness to dopamine [17], these cells maintain the ability to synthesize and release prolactin in response to many of the physiological regulators, including TRH [18,19] and VIP [20,21]. These cells therefore have been used widely as a model system to study prolactin secretion and gene regulation.

Cholera toxin (CTX) catalyses ADP-ribosylation of the G_s protein [22,23], thereby inhibiting its GTPase activity; this in turn locks the G_s protein in its active conformation, which activates adenylate cyclase persistently. In the pituitary-growth-hormone secreting cells, the inactivation of GTPase due to mutation of G_s led to persistent elevation of intracellular cyclic AMP and aberrant cell growth [24]. It was reported that CTX transgene in mice caused pituitary hyperplasia and gigantism [25], suggesting that cyclic AMP may be the mediator of growth-hormone secretion and cell proliferation in somatotrophs. Sustained stimulation with CTX, however, has been found to result in G_s -protein down-regulation in certain cell systems. In GH_3 cells, continuous stimulation with CTX induced the degradation of G_s protein [26]. Relatively long-term CTX treatment may also affect G-proteins other than G_s , for example, exposure of striatal neurons to CTX caused a decrease in the subsequent pertussis-toxin-catalysed ADP-ribosylation of G_i and G_o [27]. Although the effect of CTX on constituents of the G_s -mediated signalling

pathway in GH_3 cells was documented, cellular response such as prolactin secretion in response to persistent CTX stimulation has not been examined. In this paper, we studied prolactin secretion in response to prolonged stimulation with low concentrations of CTX and examined its correlation with activities of the G_s -protein-mediated signalling components. Our findings indicate that attenuation of $G_s\alpha$ (α -subunit of G_s)-protein expression was preceded by an up-regulation which probably resulted from an increase in $G_s\alpha$ mRNA accumulation in the early phase of CTX treatment. Analysis of time courses of prolactin secretion, cyclic AMP accumulation and G_s -protein expression indicates a correlation between these events. Our results suggest that levels of $G_s\alpha$ -protein expression may be responsible for cellular responses in response to prolonged CTX exposure.

MATERIALS AND METHODS

The DNA probe for $G_s\alpha$ subunit and anti- $G_s\alpha$ antibody (129) were obtained from Dr. Craig C. Malbon, SUNY at Stony Brook. Purified prolactin and prolactin antiserum were from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Baltimore, MD, U.S.A. GH_3 clonal rat pituitary cell line was from American Type Culture Collection, Rockville, MD, U.S.A. [35 S]Methionine (1000 Ci/mmol), [α - 32 P]ATP (3000 Ci/mmol) and cyclic [3 H]AMP (44 Ci/mmol) were from Amersham (U.K.). 32 P[NAD] $^+$ (10–50 Ci/mmol) and 125 I-prolactin (rat) were from Du Pont–New England Nuclear, Boston, MA, U.S.A. Pertussis toxin, VIP, TRH and forskolin were from Sigma Chemical Co., St Louis, MO, U.S.A. Ham's F-10 medium, fetal bovine serum and horse serum were from Gibco Laboratories, Gaithersburg, MD, U.S.A.

Cell culture

GH_3 cells were cultured in Ham's F-10 medium supplemented with 15 % horse serum and 2.5 % fetal bovine serum. Cells were

maintained under air/CO₂ (19:1) in a humidified chamber at 37 °C. Monolayer culture was maintained in a Falcon Primaria dish or a culture flask pretreated with 0.15% (w/v) Type I collagen.

Radioimmunoassay for prolactin

GH₃ cells were stimulated with CTX (5 ng/ml) for various time intervals up to 24 h. Cells were washed and then treated in the absence or presence of TRH or VIP for 30 min. Media were collected for prolactin radioimmunoassay. Cells were washed and resuspended in PBS and lysed by repeated freeze-thawing 3 times. Amounts of prolactin in the cell extract and medium were measured by radioimmunoassay with protocols supplied by the National Hormone and Pituitary Program. The assay was performed in triplicate on all samples.

Radioimmunoprecipitation

GH₃ cells were treated with CTX (5 ng/ml) for the designated time intervals. At 30 min before labelling, cells were switched to methionine-free Dulbecco's modified Eagle's medium. The cells were then labelled with [³⁵S]methionine (100 µCi/ml) in the same medium for 1 h. Medium and cells were collected separately for prolactin immunoprecipitation by the methods described by Antoniades and Pantazis [28]. Briefly, cells were washed three times with PBS containing 0.1 mM phenylmethanesulphonyl fluoride (PMSF) and lysed with buffer [0.01 M Na₂HPO₄, 0.15 M NaCl, 0.01% (v/v) Triton X-100, 0.5% (w/v) SDS, 0.2% (w/v) NaN₃, pH 7.25]. After centrifugation at 10000 g (*r*_{av}, 7 cm) for 30 min, Protein A-coupled Sepharose was added to the supernatant and the precipitate was discarded to eliminate the non-specific binding. The supernatant was incubated with anti-prolactin antibodies (anti-rPRL-S-9) for 16–18 h at 4 °C on a rocker platform. Protein A-Sepharose was then added, and the reaction proceeded for 2 h. The immunoprecipitates were then analysed by electrophoresis on an SDS/10%-polyacrylamide gel, and protein bands were revealed by autoradiography.

Cyclic AMP assay

Cells were lysed with 10% trichloroacetic acid, and centrifuged at 10000 g (*r*_{av}, 9 cm) for 10 min at 4 °C. After being separated from trichloroacetic acid and other nucleotides by chromatography on Dowex-50W resin (H⁺ form, 200–400 mesh), cyclic AMP was measured by radioimmunoassay (RIA kit from New England Nuclear) as described previously [29].

ADP-ribosylation

GH₃ cells were incubated in the presence of CTX for various time intervals. Cells were washed three times with F-10 medium and suspended in 10 mM EDTA/250 mM sucrose/20 mM Tris/HCl containing 40 µM leupeptin, 0.1 mM phenylmethanesulphonyl fluoride and 1 mM dithiothreitol, pH 7.4. Cells were disrupted three times with a Microson ultrasonic cell disruptor (Heat Systems-Ultrasonics Inc., Farmingdale, NY, U.S.A.) at 40% power output for 15 s at 4 °C. Plasma membranes were obtained by centrifuging at 10000 g (*r*_{av}, 7 cm) for 30 min at 4 °C. ADP-ribosylation of the membranes with CTX (100 µg/ml) or pertussis toxin (10 µg/ml) was performed by treating membranes with [³²P]NAD⁺ (0.1 µCi/µl) at 37 °C for 30 min as described by Wang et al. [30]. The reaction mixture was separated by

electrophoresis on an SDS/10% polyacrylamide gel. ³²P-protein bands were assessed by autoradiography.

Western-blot analysis

Membranes were isolated as described above and separated on an SDS/10%-polyacrylamide gel. After electrophoresis, the proteins were transferred electrophoretically (100 V, 2 h) to a nitrocellulose paper. The immunoblotting was performed as described in [31], except that immuno complexes were made visible by colour reaction with rabbit IgG coupled to alkaline phosphatase obtained from Promega Corp. (Madison, WI, U.S.A.). The relative intensity of the bands was scanned with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RNA preparation and Northern-blot analysis

Cytosolic RNA was isolated by the methods of White et al. [32]. Cells were harvested, washed twice with PBS and suspended in 10 mM Tris/HCl, pH 7.0, containing 1 mM EDTA and 10 mM vanadylribonucleoside complexes. The cells were chilled on ice and, while vortex-mixing, 5% Nonidet P-40 was added to a final concentration of 1%. After 10 min, the mixture was centrifuged and the supernatant extracted with an equal volume of phenol. For Northern blotting, RNA was denatured with formamide/formaldehyde and applied at 20 µg per lane to a 1.2%-agarose gel containing formaldehyde for electrophoresis. RNA was transferred to nitrocellulose paper, the DNA probe was radio-labelled with [α -³²P]ATP by random priming, and hybridization was performed as described by Meinkoth and Wahl [33]. The radioactivity was detected by exposing the nitrocellulose paper to a Kodak X-ray film for a suitable length of time, and the extent of hybridization was quantified by scanning with a Personal Densitometer.

RESULTS

Effect of sustained CTX stimulation on prolactin secretion and synthesis

Earlier studies in GH₃ cells indicated that CTX at 1 µg/ml induced a decline in G_sα levels. The consequence of the G_sα degradation on cellular responses such as prolactin secretion was, however, not pursued. We examined the effect of chronic CTX exposure on prolactin secretion in GH₃ cells. Cells were subjected to a low concentration of CTX (5 ng/ml) for various lengths of time up to 24 h. At the end of the treatment, cells were washed and incubated in the absence of CTX for 30 min, and prolactin concentrations in the medium and within cells determined separately by radioimmunoassay. In one representative experiment utilizing 6 × 10⁵ cells per assay point, prolactin levels in the medium increased from 13 ng/ml in the unstimulated cells to 34 ng/ml at 1 h after stimulation; the level then slowly fell to 23 ng/ml after 24 h in the continuous presence of CTX (results not shown). To exclude the possibility that the increase in prolactin levels in the medium was merely a reflection of elevated prolactin synthesis under the influence of CTX, percentage secretion (medium prolactin/total prolactin) was used to designate the changes in prolactin release. As indicated in Figure 1 (○), prolactin secretion increased initially, reached a peak at 3 h, and declined gradually. To explore the mechanism underlying this biphasic prolactin secretion, we tested whether the hormone-stimulated prolactin secretion was altered in the toxin-treated cells. VIP and TRH were chosen since these two neuropeptides are known to stimulate prolactin secretion in GH₃ cells. After

Figure 1 Effect of CTX on prolactin secretion

GH₃ cells were treated with 5 ng/ml CTX for the designated times, then washed and stimulated with 0.01 μ M VIP (□), 0.1 μ M TRH (●) or no addition (○) for 30 min. Cells and medium were collected separately and their prolactin levels determined by radioimmunoassay. Prolactin secretion is expressed as the ratio of medium prolactin to total prolactin (% of total); values are means $\pm$ S.E.M. ($n = 4$).

Figure 2 Effect of CTX on prolactin synthesis

GH₃ cells were treated with 5 ng/ml CTX for various time intervals as indicated below. Before harvesting, cells were incubated with [³⁵S]methionine (100 μ Ci/ml) for 1 h; medium and cells were then collected separately. The cells were lysed before immunoprecipitation with anti-prolactin antiserum or normal rabbit serum as described in the Materials and methods section. The immune complexes from medium (a) and cells (b) were analysed on an SDS/10%-polyacrylamide gel, and radiolabelled protein bands were revealed by fluorography. The position of the 23 kDa prolactin is indicated at the right. Numbers at the left are molecular-mass markers in kDa. Lanes are: 1, 0 h; 2, 1 h; 3, 3 h; 4, 6 h; 5, 12 h; 6, 24 h; 7, immunoprecipitation with normal rabbit serum.

incubation in the presence of 5 ng/ml CTX for various times up to 24 h, cells were washed and stimulated with VIP (□) or TRH (●) for 30 min, and prolactin secretion was determined. As can be seen from the gap between the two curves composed of ● and ○ symbols in Figure 1, the magnitude of TRH-stimulated secretion was constant and additive with activation by CTX throughout the 24 h period of CTX pretreatment. By contrast, the ability of VIP to stimulate prolactin secretion varied with CTX pretreatment times (compare the gaps between □ and ○

Figure 3 Effect of CTX on intracellular cyclic AMP levels

GH₃ cells were treated with CTX (5 ng/ml) for the designated time intervals, medium was then removed by aspiration, and cells were lysed with 10% trichloroacetic acid and analysed for cyclic AMP concentrations as described in the Materials and methods section. Values are means $\pm$ S.E.M. ($n = 3$).

symbols); at 30 min after toxin treatment, the cells are still fully responsive to VIP. However, after 3 h of CTX pretreatment, VIP could no longer elevate prolactin secretion above CTX-stimulated levels. These findings indicate the lack of responsiveness to VIP, but not to TRH, in the prolonged-toxin-conditioned cells.

The possibility that CTX stimulated prolactin synthesis in GH₃ cells was also examined. After treatment with CTX for various periods of times, GH₃ cells were labelled with [³⁵S]methionine, prolactin released into the medium and remaining within cells was immunoprecipitated with specific antibodies. When equal portions of medium and cellular extract were analysed by SDS/PAGE, it was evident that the newly synthesized prolactin increased both in the medium (Figure 2a) and inside cells (Figure 2b) during the course of toxin treatment. Maximal synthesis was attained between 3 and 6 h after CTX addition; attenuated CTX-induced synthesis was observed at 24 h of continuous stimulation. It is evident that, besides its effect on secretion, CTX also increased the rate of prolactin synthesis.

Effect of CTX on the intracellular cyclic AMP levels

Despite the continuous presence of CTX, prolactin secretion followed a biphasic time response, increasing initially and attenuated after 3 h (Figure 1, ○ symbols). To study factors governing this attenuated response during the course of toxin treatment, intracellular cyclic AMP levels were analysed. Cells were treated with 5 ng/ml CTX for various times up to 24 h, and cyclic AMP levels determined. As shown in Figure 3, cyclic AMP levels followed a biphasic response to the time of CTX treatment. It increased initially, approached a maximum at 3 h and declined afterwards. Throughout the 24 h of CTX treatment, however, levels of cyclic AMP were maintained above the basal concentration. The tight correlation between CTX-induced changes in cyclic AMP levels and prolactin secretion indicates that CTX-stimulated prolactin secretion may be mediated by cyclic AMP.

The possibility that prolonged CTX exposure would induce a change in adenylate cyclase activity was next studied by analysing the effect of forskolin, a direct activator of the enzyme, on cyclic AMP production. Cells were pretreated with CTX for various times; at the end of the incubation they were stimulated with forskolin for 5 min, and the intracellular cyclic AMP concentrations were determined. Figure 4 indicates that the forskolin-

Figure 4 Effect of CTX on forskolin-stimulated cyclic AMP production

GH₃ cells were treated with CTX (5 ng/ml) for the indicated time intervals. Cells were washed and stimulated with forskolin (1 μ M) for 5 min, and then lysed with 10% trichloroacetic acid and the intracellular cyclic AMP concentrations were determined as described in the Materials and methods section. Values are means $\pm$ S.E.M. ($n = 3$).

Figure 5 Effect of CTX on the toxin-catalysed ADP-ribosylation *in vitro*

GH₃ cells were treated with CTX (5 ng/ml) for various time intervals. Membranes were isolated and incubated with 100 μ g/ml CTX (a) or 10 μ g/ml pertussis toxin (b) in the presence of [³²P]NAD⁺ for 30 min as described in the Materials and methods section. They were then analysed on a SDS/10%-polyacrylamide gel, and the radioactivity was detected by autoradiography. Lanes are: 1, 0 h; 2, 1 h; 3, 3 h; 4, 6 h; 5, 12 h; 6, 24 h; 7, control cells incubated in the absence of toxins during the ADP-ribosylation reactions *in vitro*. Arrows on the right indicate positions for ADP-ribosylated bands catalysed by (a) CTX and (b) pertussis toxin. Numbers on the left are molecular-mass markers in kDa.

stimulated cyclic AMP production increased within the first 3 h of toxin treatment, and declined after chronic toxin exposure. The fact that similar time-response curves were obtained for prolactin secretion, cyclic AMP accumulation and adenylate cyclase activity in the CTX-treated cells points to a tight coupling between these events.

Effect of CTX on the subsequent toxin-catalysed ADP-ribosylation

The possible involvement of G-proteins in the attenuated cellular responses following CTX stimulation was investigated. The

Figure 6 Effect of CTX on the immunoreactive G_α protein levels

GH₃ cells were treated with CTX (5 ng/ml) for the designated times, and membranes were isolated and separated on a 10%-polyacrylamide gel. The proteins were transferred to a nitrocellulose paper, and immunoblotting was performed with G_α-specific antibody (a). The immunoreactive proteins were detected by colour reaction with rabbit IgG coupled to alkaline phosphatase. The arrow at the right indicates the position of the 52 kDa G_α protein; numbers at the left are molecular-mass markers in kDa. The relative amounts of the immunoreactive 52 kDa protein as determined by densitometry of the representative immunoblots are shown in (b); values are expressed as percentage of control. Two other independent experiments gave similar results.

extent of ADP-ribosylation during the course of CTX incubation was analysed. GH₃ cells were exposed to a CTX concentration of 5 ng/ml for various time periods, plasma membranes were isolated and the subsequent CTX-catalysed ADP-ribosylation *in vitro* was performed. Since G_α-protein is the major substrate of CTX in cells, as expected, prior treatment of cells with CTX would decrease its subsequent ability to ADP-ribosylate the G_α protein. This was indeed observed: as indicated in Figure 5(a), the [³²P]NAD⁺-labelled 52 kDa G_α protein decreased with time of CTX exposure, and at 3 h after stimulation ADP-ribosylation was essentially blocked. This indicates that the concentration of CTX used in the experiments was sufficient to react with cellular G_α protein. Besides G_α, CTX has been reported to ADP-ribosylate pertussis-toxin substrates in HL-60 leukaemia cells [34]. We determined if this was also the case in GH₃ cells. Membranes were prepared from cells pretreated with CTX, and ADP-ribosylated with pertussis toxin. As shown in Figure 5(b), the intensity of the pertussis-toxin-catalysed ADP-ribosylated band (G_α) was decreased significantly 6 h after CTX exposure. It is possible either that CTX cross-reacted with G_α, or, alternatively, there was a cross-regulation between these two G-protein-mediated pathways.

Effect of CTX on G_α protein and mRNA expression

The possibility that CTX induced changes in the amount of G_α protein was next studied. Western-blot analysis was used to determine the expression of G_α after various periods of CTX

Figure 7 Effect of CTX on the $G_s\alpha$ mRNA expression

GH₃ cells were treated with CTX (5 ng/ml) for the indicated times, and cellular RNA was isolated and Northern-blot analysis performed with 32 P-labelled $G_s\alpha$ cDNA (sp. radioactivity 8×10^8 c.p.m./ μ g) as a probe (a). The same autoradiogram was scanned with a densitometer, and results are expressed as percentage of control (b). Two other dependent experiments gave similar results.

treatment. The anti- $G_s\alpha$ antibody used was produced against a synthetic peptide corresponding to the C-terminal decapeptide (amino acid residues 386–395) of $G_s\alpha$; this region is distal in sequence to the potential CTX-catalysed ADP-ribosylation site (Arg-201). As shown in Figures 6(a) and 6(b), levels of immunoreactive $G_s\alpha$ protein were found to increase in the first 3 h of CTX (5 ng/ml) treatment. This was followed by a progressive decline; incubation with CTX for 24 h resulted in approx. 50% loss of the immunoreactive $G_s\alpha$. By analogy with previous findings using higher concentrations of CTX [26], the decreased $G_s\alpha$ expression can be attributed to the CTX-induced degradation of the protein. The elevation in $G_s\alpha$ levels found immediately after CTX stimulation was, however, not detected in the aforementioned study [26]. Northern-blot analysis was used to determine whether the increase in $G_s\alpha$ levels detected at early times after CTX treatment was caused by the enhanced $G_s\alpha$ mRNA expression. As shown (Figures 7a and 7b), there was a moderate increase in the $G_s\alpha$ mRNA accumulation in the initial 6 h of CTX treatment; this was followed by a gradual decrease. At 48 h, the $G_s\alpha$ mRNA expression was only about 40% of untreated levels (results reproduced from three independent experiments). These findings suggest that regulation at the point of gene expression may also contribute to the CTX-induced changes in $G_s\alpha$ levels.

DISCUSSION

G-proteins play key roles in transducing hormonal signals [35–37]. Evidence indicates that levels of G-proteins are also an important target for regulation (for review, see [38]). It has been reported in many cell systems that G-protein expression changes in response to the continuous presence of hormones. For example, stimulation of the adenosine A_1 receptor for 48 h in adipocytes

resulted in a 50% decrease in pertussis-toxin-catalysed ADP-ribosylation of the 41 kDa $G_i\alpha$ protein [39]. In GH₃ cells, glucocorticoid was shown to increase the cellular content of $G_s\alpha$ mRNA and membrane $G_s\alpha$ protein expression [40]. We have used CTX as a tool to study prolactin secretion under the influence of continuous $G_s\alpha$ stimulation. We showed that persistent stimulation of GH₃ cells with CTX resulted in a biphasic time response in prolactin release; analysis of underlying mechanisms indicates that similar time responses were also observed for $G_s\alpha$ protein expression, and forskolin-stimulated and basal cyclic AMP production. These data imply that G_s protein levels are the primary control for the CTX-stimulated second-messenger changes and prolactin secretion.

We have also analysed the VIP-stimulated cyclic AMP production in the CTX-conditioned cells (results not shown), and again observed a tight coupling between the VIP-stimulated prolactin secretion and cyclic AMP production. These two events were both blocked completely after 3 h of CTX treatment; at this time, ADP-ribosylation *in vitro* of the $G_s\alpha$ protein approached a minimum (Figure 5a), whereas the amount of immunoreactive $G_s\alpha$ remained elevated (Figure 6). These findings suggest that loss of cellular response to VIP stimulation is probably due to the unavailability of receptor-coupled G_s protein, and not to the loss of the protein itself. It is conceivable that, on activation by ADP-ribosylation, $G_s\alpha$ dissociates from the receptor, rendering it unresponsive to VIP. In contrast, the effect of TRH on either the prolactin secretion (Figure 1) or InsP_3 production (results not shown) was not altered by CTX; it appears that sustained stimulation of the G_s -protein-mediated signalling pathway does not interact with the TRH-induced cellular response.

It was shown by Chang and Bourne [26] that CTX induced a degradation of $G_s\alpha$ protein. At concentrations of 1 μ g/ml, there was a progressive decrease in the immunoreactive $G_s\alpha$ after 1 h of CTX treatment; by 8 h, more than 90% of the protein was degraded. Despite loss of a substantial fraction of $G_s\alpha$, however, adenylate cyclase activity remained elevated. These results led to the suggestion that, under the influence of CTX, the amount of $G_s\alpha$ protein may be in larger excess than is required for maximal stimulation of adenylate cyclase. We have, however, found that, with low concentrations of CTX, the expression of $G_s\alpha$ protein was quite distinct from those found previously [26] utilizing higher CTX concentrations. The immunoreactive $G_s\alpha$ protein was initially up-regulated; down-regulation proceeded afterwards with a slower rate and to a much smaller extent than in cells treated with high dosages of CTX. About 50% of the immunoreactive $G_s\alpha$ protein was still detectable after 24 h in the continuous presence of CTX. Moreover, the forskolin-stimulated cyclic AMP production paralleled the $G_s\alpha$ protein levels, suggesting that $G_s\alpha$ was not in excess. Although explanations of these discrepancies were not readily available, it cannot simply be attributed to insufficient quantities of CTX for reacting with $G_s\alpha$. As shown by the ADP-ribosylation *in vitro*, at concentrations used in the present study essentially all of the cellular $G_s\alpha$ protein was ADP-ribosylated during the pretreatment (Figure 5). It is worth mentioning that, in GH₃ cells stimulated with glucocorticoid, there was a parallel increase in the expression of $G_s\alpha$ protein and adenylate cyclase activity [40]; supporting that $G_s\alpha$ was limiting.

In addition to the regulation of the extent of its protein expression, several lines of evidence indicate that G-proteins could also be controlled at the level of mRNA expression. For example, in neuroblastoma $\times$ glioma NG108-15 cells, chronic exposure to ethanol caused a decrease in the $G_s\alpha$ protein levels which was accompanied by a decreased $G_s\alpha$ mRNA accumulation. This suggests that transcriptional control might be

involved in regulating G-protein levels [41]. Persistent activation of the adenylate cyclase pathway by the adenosine agonist phenylisopropyladenosine promoted an increase in the steady-state mRNA levels of $G_{i\alpha}$; however, an associated elevation in the protein expression of $G_{i\alpha}$ was not observed, possibly due to the simultaneous decrease in its half-life [42]. Hadcock et al. [43] have shown that sustained activation of adenylate cyclase by forskolin in S49 mouse lymphoma cells increased the transcript level of $G_{i\alpha 2}$; this increase was also reflected in its protein expression. The present findings that CTX caused a parallel up-regulation in both the $G_{s\alpha}$ mRNA and protein levels in the early hours of the treatment suggest that elevated mRNA accumulation may be the primary basis for increased $G_{s\alpha}$ protein expression. Similar patterns of $G_{s\alpha}$ protein and mRNA expression were also shown in S49 cells stimulated persistently with forskolin [43]; it appears that cyclic AMP is mediating the $G_{s\alpha}$ mRNA expression in these cell systems. After the initial up-regulation, both the mRNA and protein levels of $G_{s\alpha}$ started to decrease; there was, however, a discrepancy in the onset of decline between these two events (Figures 6 and 7). The decline in the amount of $G_{s\alpha}$ protein preceded that of the $G_{s\alpha}$ mRNA accumulation; between 3 and 6 h after CTX stimulation, $G_{s\alpha}$ mRNA expression continued to increase, whereas its protein expression started to fall. It seems that, after a period of continuous CTX exposure, increased protein turnover may dominate regulation of gene expression in controlling $G_{s\alpha}$ protein levels.

The findings that sustained activation of CTX decreased the available substrate for the subsequent pertussis-toxin-catalysed ADP-ribosylation was not totally unexpected. Similar results have also been observed in striatal neurons; both the pertussis-toxin-catalysed ADP-ribosylation of the α -subunits of G_i and G_o decreased after persistent CTX activation ($5 \mu\text{g/ml}$, 22 h) [44]. It is possible that, in GH_3 cells, CTX could ADP-ribosylate pertussis-toxin substrates. Alternatively, continuous stimulation of G_s protein would induce the down-regulation of pertussis-toxin-sensitive G-protein. This may indicate cross-regulation between different G-protein-mediated signalling pathways in GH_3 cells.

In conclusion, we have provided evidence that, in GH_3 cells under the prolonged stimulation of low concentrations of CTX, G-protein expression was regulated at multiple levels in a complex manner. The initial up-regulation of the $G_{s\alpha}$ mRNA transcript probably is responsible for the increased amount of immunoreactive $G_{s\alpha}$ protein. Subsequently CTX-induced $G_{s\alpha}$ -protein degradation dominated the regulatory process, resulting in down-regulation of $G_{s\alpha}$ protein level and attenuated cellular responses, including cyclic AMP production and prolactin secretion.

We thank Dr. C. C. Malbon for kindly providing us with the DNA probe and antiserum for $G_{i\alpha}$. This work was supported by grant NSC81-0420-B010-543 from the National Science Council, Republic of China.

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