Intoxication of Cultured Cells by Cholera Toxin: Evidence for Different Pathways When Bound to Ganglioside G_{M1} or Neoganglioproteins

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ABSTRACT: We previously reported that when the oligosaccharide of ganglioside G_{M1} is covalently attached to cell surface proteins of G_{M1}-deficient rat glioma C6 cells, the cells bind large amounts of cholera toxin (CT) but their cAMP response to CT is not enhanced [Pacuszka, T., & Fishman, P. H. (1990) *J. Biol. Chem. 265*, 7673–7668]. We now report that when such cells were exposed to CT in the presence of chloroquine, an acidotropic agent, they accumulated cAMP. This raised the possibility that CT bound to cell surface "neoganglioproteins" may be entering the cells through a different pathway from that of CT-bound G_{M1}. To further explore this phenomenon, we covalently attached G_{M1} oligosaccharide to human transferrin (Tf). The modified protein (G_{M1}OS–Tf) bound with high affinity to Tf receptors on HeLa cells and increased the binding of CT to the cells. The bound CT, however, was unable to activate adenylyl cyclase as measured by cyclic AMP accumulation. By contrast, treatment of HeLa cells with G_{M1} increased both CT binding and stimulation of cyclic AMP accumulation. Control cells and cells treated with either G_{M1} or G_{M1}OS–Tf were exposed to CT in the presence of chloroquine. Whereas chloroquine had little or no effect on the response of control or G_{M1}-treated cells to CT, it made the cells treated with G_{M1}OS–Tf responsive to the toxin. Our results indicate that CT bound to its natural receptor G_{M1} enters the cells through a pathway different from that of toxin bound to neoganglioproteins.

Cholera toxin (CT),[1] an enterotoxin produced by *Vibrio cholerae* is a persistent activator of adenylyl cyclase in vertebrate cells [reviewed in Fishman (1990)]. CT is composed of A and B components. The A component consists of two peptides linked by a disulfide bond, A_1 and A_2. The A_1 peptide is an ADP-ribosyltransferase whose target is the stimulatory G protein (G_s) of the adenylyl cyclase complex; transfer of ADP-ribose from NAD to the α subunit of G_s results in its persistent activation (Moss & Vaughan, 1988; Gilman, 1987). The B component is composed of five identical subunits and binds to specific receptors on the cell surface which have been identified as the ganglioside G_{M1} (Fishman, 1982, 1990). CT recognizes and binds to the oligosaccharide moiety of G_{M1}, each B component binding to five G_{M1}OS (Fishman et al., 1978).

Although many details of CT action have been elucidated (Fishman, 1990), it is still unclear as to how the A component (or more specifically the A_1 peptide) gains access to $G_{s\alpha}$ on the cytoplasmic side of the cell membrane. One model envisions the A component directly penetrating the lipid bilayer of the membrane (Fishman, 1990). Others have proposed that the holotoxin undergoes endocytosis through noncoated membrane invaginations (Tran et al., 1987) and is processed in an acidic endosomal compartment from which the A_1 peptide is formed and released (Janicot & Desbuquis, 1987; Janicot et al., 1988, 1991). The latter studies were done in rat liver or isolated hepatocytes and mainly are based on the ability of chloroquine to inhibit the generation of the A_1 peptide and the activation of adenylyl cyclase by CT [see also Houslay and Eliott (1981)].

Recently, we developed a method to generate cell surface neoganglioproteins by attaching G_{M1}OS to sulfhydryl groups on cell surface proteins of viable cells (Pacuszka & Fishman, 1990). We used G_{M1}-deficient rat glioma C6 cells and found that although the modified cells bind high levels of CT, its binding did not result in adenylyl cyclase activation. In the present study, we found that in the presence of chloroquine CT bound to such neoganglioproteins was able to activate the cyclase in C6 cells. In addition, we synthesized G_{M1}OS–Tf by covalently attaching G_{M1}OS to human Tf. The modified Tf was able to bind to Tf receptors on HeLa cells and promote the binding of CT. Such bound CT, however, was inactive unless chloroquine was present. We propose that, at least in nonhepatic cells, CT bound to its natural receptor, G_{M1}, enters the cells by a pathway different from that of receptor-mediated endocytosis used by transferrin (Hanover & Dickson, 1985).

Experimental Procedures

Materials. Cholera toxin and its B subunit were purchased from List Biological Laboratories (Campbell, CA) and for radioiodination from ICN Biochemicals (Cleveland, OH). The recombinant B subunit of *Escherichia coli* heat-labile enterotoxin was a generous gift from Dr. John Clements, Tulane University Medical Center. 3-Isobutyl-1-methylxanthine, iron-free and diferric human transferrin, and chloroquine were from Sigma Chemical Co. (St. Louis, MO). Disuccinimidyl

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[1] Abbreviations: CT, cholera toxin; G_{M1}, II^{3}Neu5Ac-GgOse$_4$Cer; G_{M1}OS, II^{3}Neu5Ac-GgOse$_4$; G_{M1}OSNH$_2$, 1-amino-1-deoxyglucitol derivative of G_{M1}OS; Tf, transferrin; G_{M1}OS–Tf, G_{M1}OS-modified transferrin; G_s, stimulatory G protein of adenylyl cyclase; DMEM/HEPES, Dulbecco's minimal essential medium buffered with 25 mM HEPES; EMEM/HEPES; Eagle's minimal essential medium buffered with 25 mM HEPES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

suberate was from Pierce Chemical Co. (Rockfort, IL). ^{125}I-Labeled diferric human transferrin (0.80 μCi/μg) was from Du Pont/NEN (Boston, MA). The preparations of G_{M1}, $G_{M1}OS$, and $G_{M1}OSNH_2$ have been described (Pacuszka & Fishman, 1990).

Synthesis of $G_{M1}OSNH$–Succinimidyl Suberate. The procedure was similar to that described for the coupling of $G_{M1}OSNH_2$ to succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carbonate (Pacuszka & Fishman, 1990). Briefly, $G_{M1}OSNH_2$ (1 μmol) and disuccinimdyl disuberate (30 μmol) were dissolved in 0.5 mL of anhydrous dimethylformamide containing 0.1% triethylamine, stirred under argon for 30 min at room temperature, and dried under argon. The residue was dissolved in 0.5 mL of water and extracted 5 times with 1-mL portions of chloroform. The water phase was dried under a stream of nitrogen and used promptly.

Synthesis of $G_{M1}OS$-Modified Transferrin. Transferrin was saturated with iron as described by van Renswoude et al. (1982). Briefly, 8 mg of transferrin was dissolved in 1 mL of 200 mM HEPES/1 mM $NaHCO_3$ (pH 8.0) to which 25 μL of 100 mM disodium nitrotriacetate/12.5 mM $FeCl_3$ was added. After 30 min at 37 °C, the diferric transferrin was purified on a Pharmacia PD-10 column equilibrated and eluted with 140 mM NaCl/2 mM HEPES, pH 7.6. The fractions containing transferrin were pooled and added directly to the flask containing $G_{M1}OSNH$–succinimidyl suberate. After 1 h at room temperature with occasional stirring, the reaction mixture was kept at 4 °C overnight, concentrated to 0.7 mL by ultrafiltration, and purified on a PD-10 column as described above. The fractions containing transferrin were pooled, dialyzed against the same buffer overnight at 4 °C, and stored in small portions (3.3 mg/mL) at −80 °C.

Characterization of $G_{M1}OS$-Modified Transferrin. On the basis of protein content, over 70% of the transferrin was recovered as $G_{M1}OS$–Tf. The 465 nm/280 nm absorbance ratio was 0.046 which indicated that the modified transferrin was iron-saturated (van Renswoude et al., 1982). Sialic acid content increased by 3.4 mol/mol. To further confirm the covalent incorporation of $G_{M1}OS$ into transferrin, a double diffusion-in-gel microplate method was used (Holmgren et al., 1974). Briefly, the center well was filled with CT and the ring wells were filled with transferrin, modified transferrin, and G_{M1}. The latter two compounds, but not the former, gave strong precipitation lines in the agar gel. Finally, $G_{M1}OS$–Tf was analyzed by a Western blotting technique with ^{125}I-CT (Pacuszka & Fishman, 1990).

Cells and Cell Culture. Rat glioma C6 cells were cultured as described (Zaremba & Fishman, 1984) except that the DMEM was supplemented with 5% Nu-Serum IV (Collaborative Research, Waltham, MA) instead of fetal bovine serum. HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (Kassis et al., 1984). For most of the experiments, cells were grown in 24- × 16-mm wells of cluster dishes. Routinely, the growth medium was removed and the cells were washed with DMEM buffered with 25 mM HEPES before being treated. Where indicated, HeLa cells were incubated with 0.2 μM B subunit for 30 min at room temperature in 0.25 mL of DMEM/HEPES to block endogenous receptors. In some experiments, HeLa cells were incubated with $G_{M1}OS$–Tf at 4 °C in 0.25 mL of EMEM/HEPES for 30 min. Both HeLa and C6 cells were treated with G_{M1} (usually 0.3 μM) for 1 h at 37 °C, and C6 cells were treated with 2 mM DDT and modified with a cross-linking derivative of $G_{M1}OS$ as described previously (Pacuszka & Fishman, 1990). After the various treatments, the cells were washed several times and used promptly for ^{125}I-cholera toxin binding and cAMP accumulation.

Table I: Effect of G_{M1} and $G_{M1}OS$ Cross-linker Treatment on Rat Glioma C6 Cells As Measured by Cholera Toxin Binding and Stimulation of cAMP Accumulation[a]

treatment	^{125}I-toxin bound (fmol/well)	CT-stimulated cAMP accumulation[b] (pmol/well)	
		−chloroquine	+chloroquine
none	3.6 ± 0.86	46.7 ± 4.8	56.9 ± 0.7
0.3 μM G_{M1}	568 ± 69.5	241 ± 15.5	369 ± 2.4
20 μM $G_{M1}OS$ cross-linker	605 ± 7.7	39.3 ± 2.6	111 ± 8.7

[a] The cells were treated without and with G_{M1} in DMEM/HEPES for 1 h at 37 °C, washed, and incubated with 2 mM DDT in phosphate-buffered saline for 3 min at room temperature. The cells were again washed and incubated with and without $G_{M1}OS$ cross-linker in Dulbecco's phosphate-buffered saline (pH 7.0 plus 5 mM glucose) for 15 min at room temperature. Finally, the cells were washed and assayed for specific ^{126}I-CT binding (5 nM for 1 h) and CT-stimulated cAMP accumulation as described under Experimental Procedures. Each 16-mm well of cells usually contained 100 μg of protein; values were corrected for variations in protein and are expressed as the means ± SD of triplicate determinations. [b] The cells were incubated in the presence and absence of 100 μM chloroquine. After an additional 30 min, the cells were washed and incubated in fresh medium containing 1 mM 3-isobutyl-1-methylxanthine plus and minus 100 μM chloroquine at 37 °C. After 5 min, cholera toxin was added (20 nM final) and the cells were incubated an additional 120 min (90 min for the G_{M1}-treated cells) and assayed for cAMP. Basal cAMP levels ranged from 0.9 to 3.0 pmol/well.

Binding of ^{125}I-Cholera Toxin and -Transferrin. Binding of ^{125}I-CT to cells in clusters was done as described (Spiegel, 1985; Pacuszka & Fishman, 1990). In some experiments, binding was done at 4 °C for 1 h in Eagle's minimal essential medium (EMEM) buffered with 25 mM HEPES. Binding of ^{125}I-Tf to cells was done as follows. The cells were incubated in 0.5 mL/well EMEM/HEPES containing 0.1% bovine serum albumin, 1 nM ^{125}I-Tf, and increasing concentrations of diferric transferrin or $G_{M1}OS$–Tf for 30 min at 4 °C. The cells then were washed 3 times with ice-cold Dulbecco's phosphate-buffered saline, dissolved in 0.5 mL of 0.5 M NaOH, and counted for radioactivity in a γ-counter.

Determination of cAMP. The treated and washed cells were incubated at 37 °C in DMEM/HEPES containing 1 mM 3-isobutyl-1-methylxanthine and 0.01% bovine serum albumin with or without 10 nM cholera toxin. After the indicated times, the medium was rapidly aspirated and cAMP was determined by a radioimmune assay (Zaremba & Fishman, 1984). When chloroquine was used, it was added at 100 μM at least 30 min before the toxin was added.

Other Methods. Sialic acid was determined by its reaction with resorcinol (Svennerholm & Fredman, 1980), and protein was determined by the method of Lowry et al. (1951).

Results

Effect of Chloroquine on CT Stimulation of Rat Glioma C6 Cells. We had shown previously that a cross-linking derivative of $G_{M1}OS$ can covalently attached to free −SH groups on cell surface proteins of rat glioma C6 cells and generate neoganglioproteins which bind CT but are nonfunctional receptors (Pacuszka & Fishman, 1990). In agreement with those studies, Table I shows that untreated C6 bound only trace amounts of ^{125}I-CT and accumulated very little cAMP in response to the toxin. Both C6 cells treated with G_{M1} or with the cross-linker bound large amounts of ^{125}I-CT, but only the G_{M1}-treated cells exhibited an increased response to the toxin. To explore the possibility that CT bound to cell surface neogangliproteins may undergo endocytosis and degradation, we exposed the cells to CT for 2 h in the presence of chloroquine (Table I). Chloroquine increased the cAMP response almost

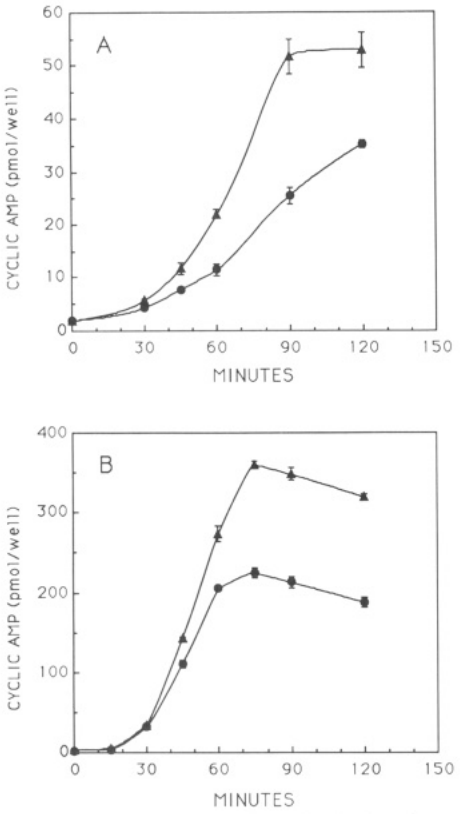

FIGURE 1: Effect of chloroquine on CT stimulation of cAMP accumulation in rat glioma C6 cells. (A) Cells were incubated for 20 min at 37 °C in the absence (●) and presence (▲) of 100 μM chloroquine, exposed to 10 nM CT for the indicated times, and assayed for cAMP content as described under Experimental Procedures. (B) The assay was the same as that in (A) except that the cells were incubated for 1 h at 37 °C with 0.3 μM G_{M1} in the absence and presence of chloroquine, washed, and exposed to CT in fresh medium with and without chloroquine for the indicated times. Values are means ± SD of triplicate determinations from a representative experiment.

3-fold over that of cells exposed to CT in the absence of the drug, and the cAMP levels were significantly higher than those in control cells exposed to CT. Chloroquine alone caused only a modest 50% increase in CT stimulation of untreated and G_{M1}-treated cells with no change in the lag period (Table I; Figure 1). The effect seemed to be somewhat variable as in other experiments the presence of chloroquine had no effect on CT stimulation (data not shown). The latter agreed with our previous observations in which we directly measured the activation of adenylyl cyclase by CT in untreated and G_{M1}-treated C6 cells (Pacuszka et al., 1991). In no experiments did we observe an inhibition by chloroquine as had been reported for hepatocytes (Housley & Elliott, 1981; Janicot et al., 1988).

Characterization of G_{M1}OS-Modified Transferrin. In order to develop a more defined pathway for receptor-mediated endocytosis of CT, we decided to covalently attach G_{M1}OS to Tf and use the modified Tf as a carrier for CT into the cells. On the basis of the increase in sialic acid content, we found that each molecule of G_{M1}OS–Tf contained 3.4 G_{M1}OS. Using a double diffusion-in-gel method, the G_{M1}OS–Tf bound to CT (see Experimental Procedures). The G_{M1}OS–Tf was separated by SDS–PAGE and transferred to nitrocellulose which was overlain with ^{125}I-CT (Figure 2). The iodotoxin detected a protein with an apparent MW of 87 000 compared to that of 80 000 for unmodified human Tf.

Binding of Transferrin and G_{M1}OS-Modified Transferrin to Cells. We initially determined the number of Tf receptors on rat glioma C6 cells and found that ^{125}I-Tf bound with a

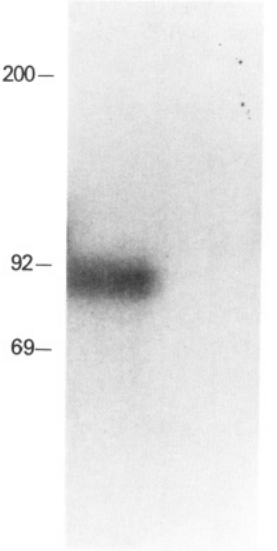

FIGURE 2: Detection of ^{125}I-CT binding to G_{M1}OS–TF by Western blotting. G_{M1}OS–TF (0.3 μg) was separated by SDS–PAGE and electrophoretically transferred to a nitrocellulose sheet which was blotted with ^{125}I-CT as described under Experimental Procedures. The locations of molecular weight markers are indicated.

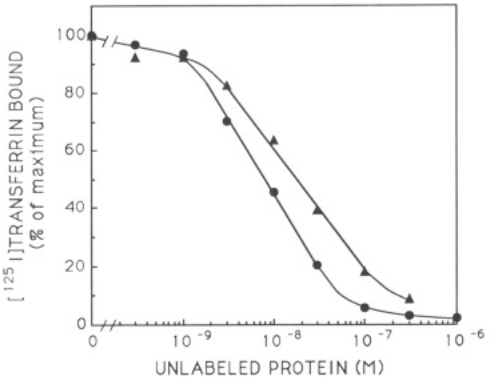

FIGURE 3: Binding of ^{125}I-Tf to HeLa cells: displacement by unlabeled Tf and G_{M1}OS–Tf. Cells were incubated at 4 °C for 30 min with 2 nM ^{125}I-Tf in the presence of the indicated concentrations of unlabeled Tf (●) or G_{M1}OS–Tf (▲), washed, and assayed for bound ^{125}I-Tf as described under Experimental Procedures. Similar results were obtained in two additional experiments.

K_d of 6.8 nM and a B_{max} of 20 fmol/well (171 fmol/mg of cell protein). Even if all these sites were occupied by G_{M1}OS–Tf, the potential increase in CT binding would be very small (see Table I). As HeLa cells bind only low levels of CT (Fishman & Atikkan, 1979, 1980), we next assayed HeLa cells for Tf receptors. From Scatchard analysis of the binding data, HeLa cells bound human diferric Tf with a K_d of 4.27 ± 0.27 nM and a B_{max} of 622 ± 73 fmol/mg of cell protein (n = 3). On the basis of competition binding (Figure 3), the G_{M1}OS–Tf recognized the Tf receptors on HeLa cells with a K_d of 11.7 ± 0.41 nM. Thus, the addition of several G_{M1}OS to Tf did not impair its ability to bind to its receptor.

We next determined whether CT would bind to the Tf receptor/G_{M1}OS–Tf complex on the cell surface. HeLa cells were incubated with G_{M1}OS–Tf at 4 °C for 30 min, washed, and assayed for ^{125}I-CT binding. As shown in Table II, toxin binding was increase 8-fold over that of control cells; for comparison, G_{M1}-treated cells bound 14.6-fold more toxin than control cells. The increased binding of CT to cells treated with G_{M1}OS–Tf was clearly being mediated through the Tf receptor as the presence of excess unmodified TF during the binding of G_{M1}OS–Tf reduced the subsequent binding of ^{125}I-CT. In

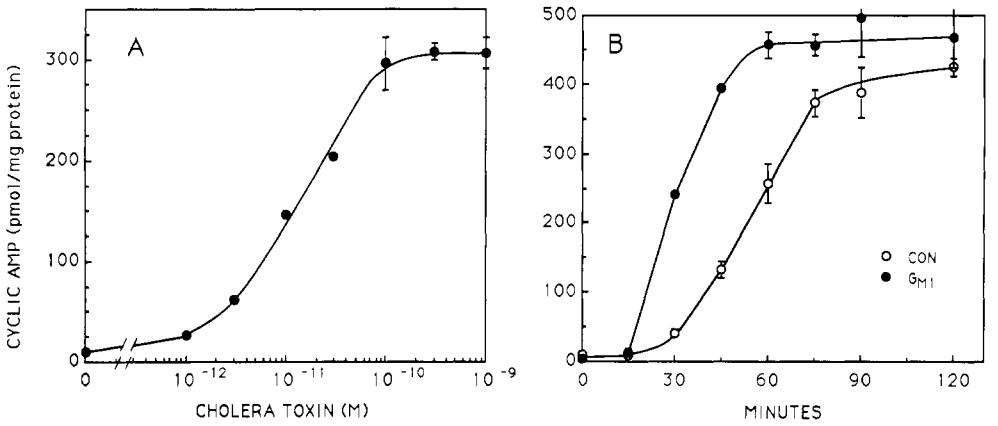

FIGURE 4: Effect of concentration and time on CT stimulation of cAMP accumulation in HeLa cells. (A) Cells were incubated with increasing concentrations of CT for 2 h at 37 °C and assayed for cAMP content as described under Experimental Procedures. (B) Untreated cells (○) or cells treated with 1 μM G_{M1} for 1 h (●) were incubated with 10 nM CT for the indicated times and assayed for cAMP content. Values are means ± SD of triplicate determinations from a representative experiment.

Table II: Effect of G_{M1} and G_{M1}OS–Tf on Binding of ^{125}I-CT to HeLa Cells[a]

treatment	^{125}I-CT bound (fmol/ mg of protein)	treatment	^{125}I-CT bound (fmol/ mg of protein)
experiment 1		experiment 2	
none	74.0 ± 6.7	0.05 μM G_{M1}OS–Tf	514 ± 46.0
0.3 μM G_{M1}	1080 ± 31.1	plus 1 μM Tf	169 ± 3.7
0.3 μM G_{M1}OS–Tf	591 ± 14.5	0.2 μM G_{M1}OS–TF plus 2 μM Tf	690 ± 16.0 / 289 ± 3.0

[a] Cells were treated with G_{M1} in DMEM/HEPES for 1 h at 37 °C or with G_{M1}OS–Tf in EMEM/HEPES containing 0.1% bovine serum albumin for 30 min at 4 °C in the presence and absence of unmodified Tf as indicated. The cells then were washed and assayed for ^{125}I-CT binding at 4 °C as described under Experimental Procedures.

addition, the amount of ^{125}I-CT binding to HeLa cells treated with G_{M1}OS–Tf was similar to the amount of ^{125}I-Tf binding.

CT Stimulation of HeLa Cells. HeLa cells are very sensitive to CT (Fishman & Atikkan, 1980). Half-maximal increases in cAMP levels occurred at 10 pM CT (Figure 4A). Although G_{M1}-treated HeLa cells bound more CT than control cells, G_{M1} treatment increased only the rate of CT stimulation and not the extent (Figure 4B). Thus, in contrast to rat glioma C6 cells, the ability to detect a stimulation by CT bound to a nonendogenous receptor may be difficult to detect in HeLa cells. In order to enhance the response through such nonendogenous receptors, we blocked the endogenous receptors with the B subunit of *E. coli* heat-labile toxin.[2] When HeLa cells were exposed first to the B subunit, it inhibited the stimulation by CT in a concentration-dependent manner (Figure 5A). The response to CT was restored by incubating the B-subunit-treated cells with G_{M1} (Figure 5B).

When B-subunit-treated cells were incubated with G_{M1}OS–Tf at 4 °C, washed, and exposed to CT for 2 h at 37 °C, there was no significant increase in cAMP levels over the basal level (20.8 ± 1.4 vs 16.3 ± 8.2 pmol/mg of protein). When chloroquine was present, however, cAMP levels increased by a small but significant amount over the corresponding basal value (51.8 ± 0.3 vs 13.4 ± 1.5). In order to simplify the manipulations, the cells were exposed to a 1/1 mixture of CT and G_{M1}OS–Tf. Under these conditions, cAMP levels rose in a time-dependent manner in the presence of

[2] We initially tried to block the endogenous G_{M1} in HeLa cells with a commerical preparation of CT B subunit, but we found only a partial inhibition of CT stimulation. As reported by Spiegel (1990), some preparations of B subunit are contaminated with A subunit. We therefore used a recombinant B subunit of *E. coli* heat-labile toxin which also has been shown to bind to G_{M1} (Moss et al., 1981).

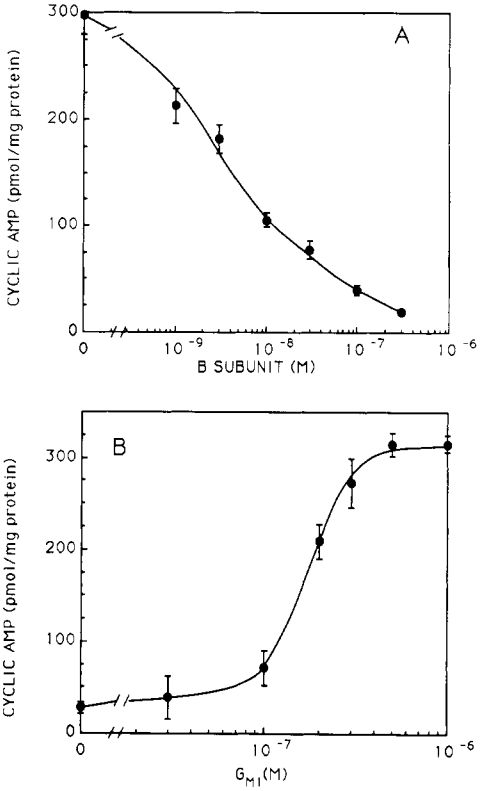

FIGURE 5: Effect of toxin B subunit and G_{M1} on response of HeLa cells to CT. (A) Cells were incubated with increasing concentrations of B subunit for 30 min at room temperature, washed, exposed to 10 nM CT for 90 min at 37 °C, and assayed for cAMP content as described under Experimental Procedures. The basal cAMP value was 9.0 ■ 1.2 pmol/mg of protein. (B) Cells were treated with 0.2 μM B subunit, washed, and incubated with increasing concentrations of G_{M1} for 1 h at 37 °C. After the cells were again washed, they were assayed for CT stimulation of cAMP accumulation as described in (A). Values are means ± SD of triplicate determinations from one of two similar experiments.

chloroquine (Figure 6A), whereas no such increase was observed in cells exposed only to CT in the presence of chloroquine. Although by 4 h cAMP levels eventually reached those observed in naive cells stimulated by CT, the response was much slower and the lag period was much longer (compare with Figure 4B).

We next confirmed that CT was stimulating the HeLa cells through G_{M1}OS–Tf bound to Tf receptors. The cells were treated with B subunit and chloroquine as usual and then exposed to G_{M1}OS–Tf and CT in the presence of unmodified

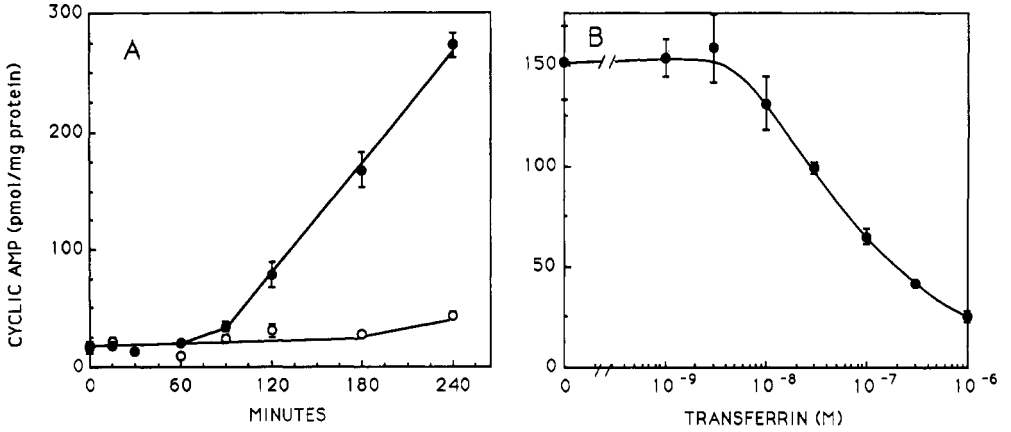

FIGURE 6: Stimulation of cAMP accumulation in HeLa by CT bound to G_{M1}OS–Tf. (A) Cells were first treated with B subunit and 100 μM chloroquine and were then exposed to 10 nM CT (○) or a mixture of 10 nM CT and 10 nM G_{M1}OS–Tf (●) for the indicated times at 37 °C and assayed for cAMP content as described under Experimental Procedures. (B) Cells treated with B subunit and chloroquine were exposed to a mixture of 10 nM CT and 10 nM G_{M1}OS–Tf in the presence of increasing concentrations of Tf for 4 h at 37 °C and assayed for cAMP content. The basal cAMP value was 20.9 ± 2.3 pmol/mg of protein. Values are means ± SD of triplicate determinations from one of two similar experiments.

Table III: Effect of Chloroquine on CT Stimulation of Control and G_{M1}-Treated HeLa Cells[a]

treatment		cyclic AMP accumulation (pmol/mg of protein)		
			+CT	
B subunit	G_{M1}	−CT	−chloroquine	+chloroquine
−	−	9.0 ± 1.2	294 ± 11.7	280 ± 17.7
−	+	7.8 ± 0.9	326 ± 8.2	325 ± 11.2
+	−	11.0 ± 3.7	9.4 ± 1.8	9.5 ± 3.4
+	+	9.4 ± 1.8	315 ± 9.0	320 ± 19.5

[a] The cells were incubated in DMEM/HEPES with and without 0.2 μM B subunit for 30 min, washed, and incubated with and without 1 μM G_{M1} for 1 h at 37 °C. The cells were washed and incubated in DMEM/HEPES containing 0.01% bovine serum albumin and 1 mM 3-isobutyl-1-methylxanthine with and without 10 nM CT. After 90 min at 37 °C, the cells were assayed for cAMP and protein as described under Experimental Procedures. Where indicated, 100 μM chloroquine was present during all the incubations.

Tf. The ability of CT to stimulated cAMP accumulation was inhibited by Tf in a concentration-dependent manner (Figure 6B). This was consistent with Tf inhibiting the binding of G_{M1}OS–Tf to Tf receptors and preventing the increased binding of ^{125}I-CT to the cells as shown above. Tf had no effect on the stimulation of naive HeLa cells by CT (data not shown).

Although chloroquine appeared to be required for the action of CT when it was bound to the G_{M1}OS–Tf/Tf receptor complex, it had little if any effect on the response of untreated or G_{M1}-treated HeLa cells to CT (Table III; Figure 7). Chloroquine even had no effect on HeLa cells when endogenous receptors were blocked by B subunit and replaced with exogenous G_{M1} (Table III). As we had observed with the C6 cells, there was some variation among experiments. In five experiments with untreated HeLa cells, chloroquine reduced the cAMP stimulation by CT to 90.7 ± 3.8% of the control and in one experiment, it was increased to 108.5%. In four experiments with G_{M1}-treated HeLa cells, chloroquine reduced the response to 90.5 ± 5.5% of the control.

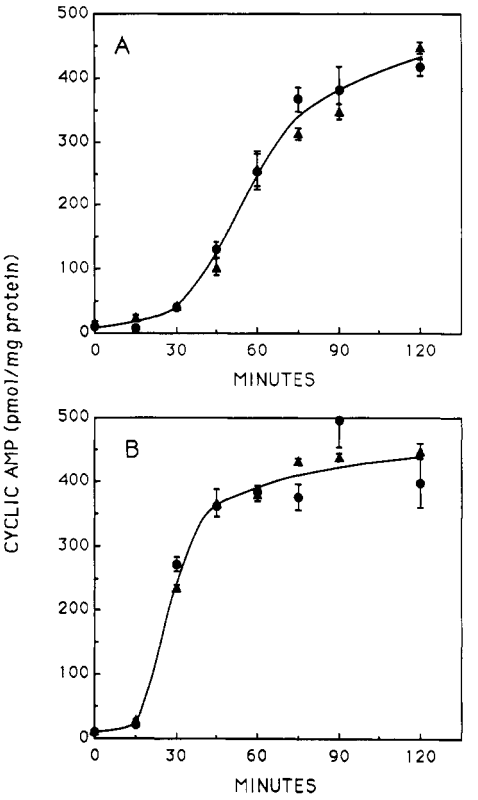

FIGURE 7: Effect of chloroquine on CT stimulation of control and G_{M1}-treated HeLa cells. (A) Cells were incubated for 20 min at 37 °C in the absence (●) and presence (▲) of 100 μM chloroquine, exposed to 10 nM CT for the indicated times, and assayed for cAMP content as described under Experimental Procedures. (B) The assay was the same as that in (A) except that the cells were incubated for 1 h at 37 °C with 1 μM G_{M1} in the absence and presence of chloroquine, washed, and exposed to CT in fresh medium with and without chloroquine for the indicated times. Values are means ± SD of triplicate determinations from a representative experiment.

Discussion

In the present study, we explored the possibility that CT bound to neoganglioproteins might enter cells through a different pathway from that of toxin bound to its native receptor, G_{M1}. Using two different approaches and two different cell lines, our results support this hypothesis. Rat glioma C6 cells expressing G_{M1}OS-modified cell surface proteins were able to bind CT but were unable to accumulate cAMP in response to the bound toxin [present study and Pacuszka and Fishman (1990)]. When these modified cells were exposed to CT in the presence of chloroquine, however, a significant cAMP response was observed. In the second approach, we synthesized a G_{M1}OS conjugate of human Tf. The G_{M1}OS–Tf was able to bind both to CT and to Tf receptors on HeLa cells and thus served as "bridge" to allow the toxin to bind to the cell surface. Again, the bound CT was unable to stimulate HeLa cells

unless the cells had been treated with chloroquine. We believe that these latter results are especially relevant as it is well-established that Tf enters the cell through receptor-mediated endocytosis (Hanover & Dickson; 1985). Furthermore, the lag period, which represents the time between toxin binding and toxin stimulation, was more than twice as long when CT was bound to Tf receptors through $G_{M1}OS$–TF as when CT was bound to G_{M1}. This difference is consistent with the toxin being internalized and processed through a different pathway.

Although chloroquine appeared to be necessary for CT bound to neoganglioproteins to be active, it had only a modest effect on the response of untreated or G_{M1}-treated rat glioma C6 and HeLa cells to the toxin.[3] At best, it enhanced the response in C6 cells by up to 50% and inhibited the response in HeLa cells by less than 10%. With neither cell line did the presence of chloroquine have any effect on the lag period (Figures 1 and 7). Our results are in marked contrast to those reporting that chloroquine inhibits CT action both in rat liver when the toxin is injected in vivo and in isolated rat hepatocytes (Houslay & Eliott, 1981; Janicot & Desbuquis, 1987; Janicot et al., 1988, 1991). In these studies, the presence of chloroquine was found to double the lag period for CT action, to reduce the response to CT by 50–80%, and to inhibit the generation of the A_1 peptide by 50%. Regarding the last point, we had previously reported that chloroquine actually increases the generation of the A_1 peptide by 2.5–4-fold in rat glioma C6 cells (Pacuszka et al., 1991). These differences may be due to differences between hepatocytes and other cell types. A major role of liver is detoxification, and hepatocytes may be undergoing more active endocytosis than the established cell lines used in our studies. On the other hand, chloroquine was found to have minimal inhibitory effects on cholera toxin stimulation of cAMP levels in Raw 264.7 macrophage/monocyte cells (Liang et al., 1990).

Our current results support our proposed model for the mechanism of action of CT (Fishman, 1990). When CT is bound to its natural receptor G_{M1}, the A component is able to penetrate into the lipid bilayer of the membrane and be reduced to generate the active A_1 peptide. The latter then catalyzes the ADP-ribosylation of $G_{s\alpha}$ at the cytoplasmic side of the membrane, which results in the persistent activation of adenylyl cyclase. We believe that the lipid moiety of G_{M1} plays an important role in promoting the penetration of the A subunit across the membrane. Using a series of neoglycolipid analogues of G_{M1} (Pacuszka et al., 1991), we had shown that $G_{M1}OS$ attached to either stearylamine or cholesterol is a more efficient toxin receptor than G_{M1}, whereas phospholipid analogues are less efficient than G_{M1}. The extreme situation appears to be derivatives in which $G_{M1}OS$ is attached to proteins. Even though CT was able to bind, it was inactive unless chloroquine was present. Chloroquine also potentiates the activity of CT bound to the phospholipid analogues (Pacuszka et al., 1991). These effects of chloroquine are consistent with the toxin entering the cells and being processed by a pathway which is different from that of the toxin bound to G_{M1}.

[3] We found that chloroquine also had minimal effects on cholera toxin action in murine neuroblastoma NB41A and Friend erythroleukemic cells, human diploid fibroblasts, intestinal tumor CaCo-2 cells, and neurotumor SK-N-MC cells.

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