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Mechanism of Action of Cholera Toxin: Studies on the Lag Period

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Summary. The lag period for activation of adenylate cyclase by choleragen was shorter in mouse neuroblastoma N18 cells than in rat glial C6 cells. N18 cells have 500-fold more toxin receptors than C6 cells. Treatment of C6 cells with ganglioside G_{M1} increased the number of toxin receptors and decreased the lag phase. Choleragen concentration also effected the lag phase, which increased as the toxin concentration and the amount of toxin bound decreased. The concentration, however, required for half-maximal activation of adenylate cyclase depended on the exposure time; at 1.5, 24, and 48 hr, the values were 200, 1.1, and 0.35 pM, respectively. Under the latter conditions, each cell was exposed to 84 molecules of toxin.

The length of the lag period was temperature-dependent. When exposed to choleragen at 37, 24, and 20 °C, C6 cells began to accumulate cyclic AMP after 50, 90, and 180 min, respectively. In G_{M1}-treated cells, the corresponding times were 35, 60, and 120 min. Cells treated with toxin at 15 °C for up to 22 hr did not accumulate cAMP, whereas above this temperature they did. Antiserum to choleragen, when added prior to choleragen, completely blocked the activation of adenylate cyclase. When added after the toxin, the antitoxin lost its inhibitory capability in a time and temperature-dependent manner. Cells, however, could be preincubated with toxin at 15 °C, and the antitoxin was completely effective when added before the cells were warmed up. Finally, cells exposed to choleragen for >10 min at 37 °C accumulated cyclic AMP when shifted to 15 °C. Under optimum conditions at 37°C, the minimum lag period for adenylate cyclase activation in these cells was 10 min. These findings suggest that the lag period for choleragen action represents a temperature-dependent transmembrane event, during which the toxin (or its active component) gains access to adenylate cyclase.

Choleragen (cholera toxin) is a persistent activator of adenylate cyclase in a wide variety of vertebrate cells [for recent reviews *see* 1, 3, 13, 34]. The initial event in toxin action is its binding to specific receptors on the cell surface that have been identified as the ganglioside G_{M1}[1] [5, 6, 11, 19, 23, 26, 28]. In intact cells, there is a discrete lag period between choleragen binding and activation of adenylate cyclase [2, 14, 22] which is not observed in disrupted cells [12]. More recently, the mechanism of the activation process in disrupted cells has been elucidated. The A_1 subunit of the toxin, which possess ADP-ribosyltransferase activity [29], can transfer ADP-ribose from NAD to a GTP-binding protein that regulates adenylate cyclase activity [4, 15, 20]. Thus, the activation of adenylate cyclase by choleragen appears to be a catalytic process analogous to the mechanism of action of diptheria toxin [13].

The intervening events between binding and activation of adenylate cyclase are still unclear. Although the lag period was extensively studied by Cuatrecasas and coworkers [1, 2, 30], it was prior to an awareness that choleragen is an enzyme and its mechanism of action involved a catalytic step. The accompanying report demonstrated that multivalent binding of choleragen to several receptors on the cell surface was time- and temperature-dependent and influenced by the density of these receptors [9]. The present study was initiated to examine the effects of receptor density on the lag period as well as the effects of time, temperature and toxin concentration.

Materials and Methods

Choleragen was purchased from Schwarz/Mann. Antiserum to choleragen was raised in a burro and was a generous gift from Dr. W.H. Habig, FDA. G_{M1} and other glycolipids were prepared as described previously [10, 31].

[1] *Abbreviations used:* ganglioside nomenclature according to Svennerholm [32] (*see* Table 1 for structures); cAMP, adenosine 3′:5′-monophosphate; MIX, 3-isobutyl-1-methylxanthine; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; PBS, phosphate-buffered saline (pH 7.4).

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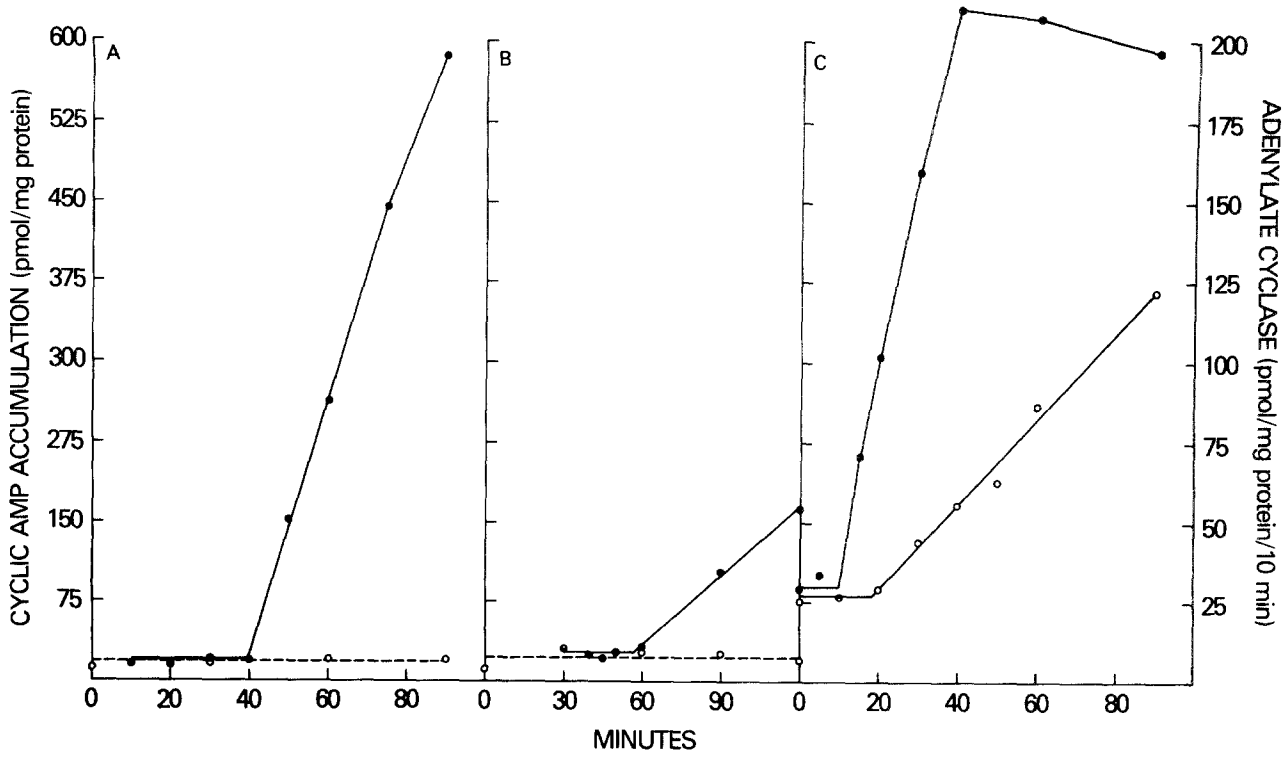


Fig. 1. Effects of choleragen on cells with high and low densities of receptors. Mouse neuroblastoma N18 (*A*) and rat glial C6 (*B*) cells cultured in 35 mm wells were incubated at 37 °C with (●) and without (○) 20 nM choleragen in 2 ml of Medium 199 containing 25 mM HEPES, 0.01% bovine serum albumin, and 0.2 mM MIX and assayed for cAMP content at the indicated times as described in Materials and Methods. (*C*) N18 (●) and C6 (○) cells suspended in 1 ml of Medium 199 containing 25 mM HEPES and 0.01% bovine serum albumin were incubated at 37 °C with 20 nM choleragen and at the indicated times assayed for adenylate cyclase activity as described in Materials and Methods. The number of toxin receptors per N18 (3.75×10^6) and C6 (7000) cell have been reported elsewhere [9]

Rat glial C6 and mouse neuroblastoma N18 cells were cultured as previously described [9]. Unless otherwise indicated, the cells were grown in T-75 flasks for adenylate cyclase assays, removed by gentle scraping with a rubber policeman in PBS, collected by centrifuging, and suspended in Medium 199 buffered with 25 mM HEPES plus 0.01% bovine serum albumin. The cells were grown in 35 mm wells of Linbro trays for cAMP accumulation experiments. Cells were treated with G_{M1} *in situ* by incubating them for 1 hr at 37 °C in Medium 199 buffered with 25 mM HEPES plus G_{M1}. The medium was then removed and the cells washed 3 times with PBS.

Activation of adenylate cyclase by choleragen and the subsequent assay of activity was as described elsewhere [9], except the cells were disrupted by standing on ice in 2 mM Tris-HCl (pH 7.4) for 5 min and then mixing vigorously on a vortex mixer. Formation of cAMP was linear for up to 15 min with up to 150 μg of cell lysate protein. Accumulation of cAMP was determined by incubating the cells with 1.5 ml of Medium 199 buffered with 25 mM HEPES for 10 min at 37 °C. Then 0.5 ml of medium containing 0.8 mM MIX and 0.04% bovine serum albumin was added, followed by choleragen where indicated. In some experiments, 2 mM MIX was added. The cells were then incubated at 37 °C for the desired time. The medium was removed and 1 ml of 0.1 M HCl was added to the cell layer [33]. The acid extract was assayed for cAMP [9], and the cell layer was digested in 1 ml of 0.2 M NaOH and assayed for protein [25]. Adenylate cyclase activity and cAMP accumulation were determined in triplicate for each experimental point and the values varied less than 10% among the triplicates.

Iodination of choleragen and binding of ^{125}I-toxin to the cells was as described elsewhere, except the filters were counted in a Beckman gamma 4000 with a counting efficiency of 80% [9]. In some experiments, binding was determined directly on cells cultured in 35 mm wells. The cells were incubated at 37 °C with ^{125}I-choleragen in 1 ml of Medium 199 containing 25 mM HEPES and 0.1% albumin for 30 min. The medium was removed and the cells washed 3 times with 2 ml of ice-cold buffer (0.15 M NaCl-5 mM Tris-HCl-0.1% albumin, pH 7.4). The cells were detached with 0.1% trypsin in PBS and the cell suspensions were analyzed for radioactivity. All values have been corrected for nonspecific binding as determined in the presence of excess unlabeled choleragen and are the mean of triplicate determinations which varied less than 10%.

Results

Effect of Receptor Density on Choleragen Action

Mouse neuroblastoma N18 cells responded more rapidly to choleragen than did rat glial C6 cells (Fig. 1.). Whether cAMP accumulation or adenylate cyclase activation was measured, the N18 cells exhibited a shorter lag period than the C6 cells. In addition, once the N18 cells began to respond to the toxin, they

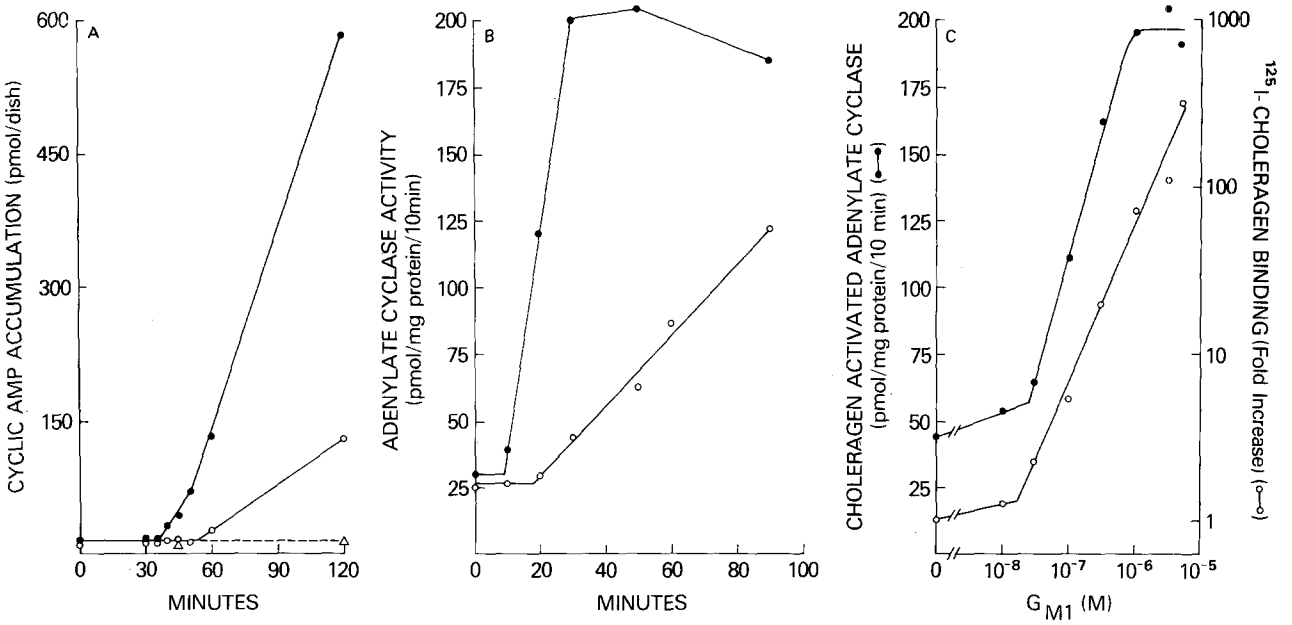


Fig. 2. Effect of G_{M1}-treatment on stimulation of C6 cells by choleragen. Rat glial C6 cells were treated with (●) and without (○, *Δ*) 5 μM G_{M1} for 1 hr, washed, incubated with (●, ○), and without (*Δ*) choleragen, and assayed for cAMP content (*A*) or adenylate cyclase activity (*B*) as described in the legend to Fig. 1. (*C*) C6 cells were treated with the indicated concentration of G_{M1} for 1 hr, washed, suspended in appropriate media, incubated with unlabeled or ^{125}I-choleragen for 30 min at 37 °C, and assayed for adenylate cyclase activity (●) or ^{125}I-toxin binding (○) as described in Materials and Methods. Binding was determined under conditions where there was an excess, saturating, concentration of iodotoxin

Table 1. Treatment of rat glial C6 cells with glycolipids: effects on choleragen binding and activity[a]

Glycolipid	Oligosaccharide structure	^{125}I-choleragen bound[b]	cAMP accumulation[c]
None		24	38.2
G_{A1}	Gal-GalNAc-Gal-Glc-	22	33.7
G_{M3}	AcNeu-Gal-Glc-	18	38.0
G_{M2}	GalNAc-[AcNeu]-Gal-Glc-	24	45.9
G_{M1}	Gal-GalNAc-[AcNeu]-Gal-Glc-	1590	325
G_{D1a}	AcNeu-Gal-GalNAc-[AcNeu]-Gal-Glc-	26	54.2
G_{D1b}	Gal-GalNAc-[AcNeu-AcNeu]-Gal-Glc-	40	37.4

[a] Rat glial C6 cells in 35 mm wells were incubated with 1 ml of Medium 199 containing 25 mM HEPES and 0.5 μM of the indicated glycolipid (0.1 μM for G_{A1}) for 1 hr at 37 °C, washed, and incubated with either 20 nM ^{125}I-choleragen for 30 min or 20 nM unlabeled choleragen for 50 min at 37 °C. Then bound iodotoxin and intracellular cAMP were determined as described in Materials and Methods. As each of the glycolipids was radiolabeled, uptake by the cells was determined and ranged from 10.3 to 19.8 pmol/mg protein.

[b] fmol/mg protein; values were corrected for nonspecific binding (cpm bound in the presence of 1 μM unlabeled choleragen).

[c] pmol/mg protein; in the absence of choleragen, cAMP content was 30.2 pmol/mg.

responded at a faster rate than did the C6 cells. Thus, with saturating levels of choleragen, adenylate cyclase became completely activated in N18 cells by 40 min when activity was still increasing in C6 cells (Fig. 1 *C*). In C6 cells, full activation required >2 hr (data not shown). Separate studies indicated that each N18 cell contained over 500-fold more toxin receptors than a C6 cell [9].

Therefore, C6 cells were treated with G_{M1} to increase their receptor numbers, and their response to choleragen was compared to untreated cells. The G_{M1}-treated cells, which now bound 350-fold more toxin than control cells, exhibited a shorter lag period and an enhanced response compared to control cells (Fig. 2). Accumulation of cAMP began in G_{M1}-treated cells after 35 min compared to 50 min in control cells; the lag period for activation of adenylate cyclase was reduced from 20 min to ~10 min. Thus, the G_{M1}-treated C6 cells were now responding as rapidly to choleragen as N18 cells. This effect was specific for G_{M1}, as treatment of C6 cells with other related glycolipids did not cause an increase in choleragen binding or responsiveness (Table 1).

In these G_{M1}-treated C6 cells, adenylate cyclase became maximally activated by 30 min (Fig. 2 *B*). The effect of varying the toxin receptors on adenylate cyclase activation at this time point is shown in Fig. 2 *C*. The number of receptors increased propor-

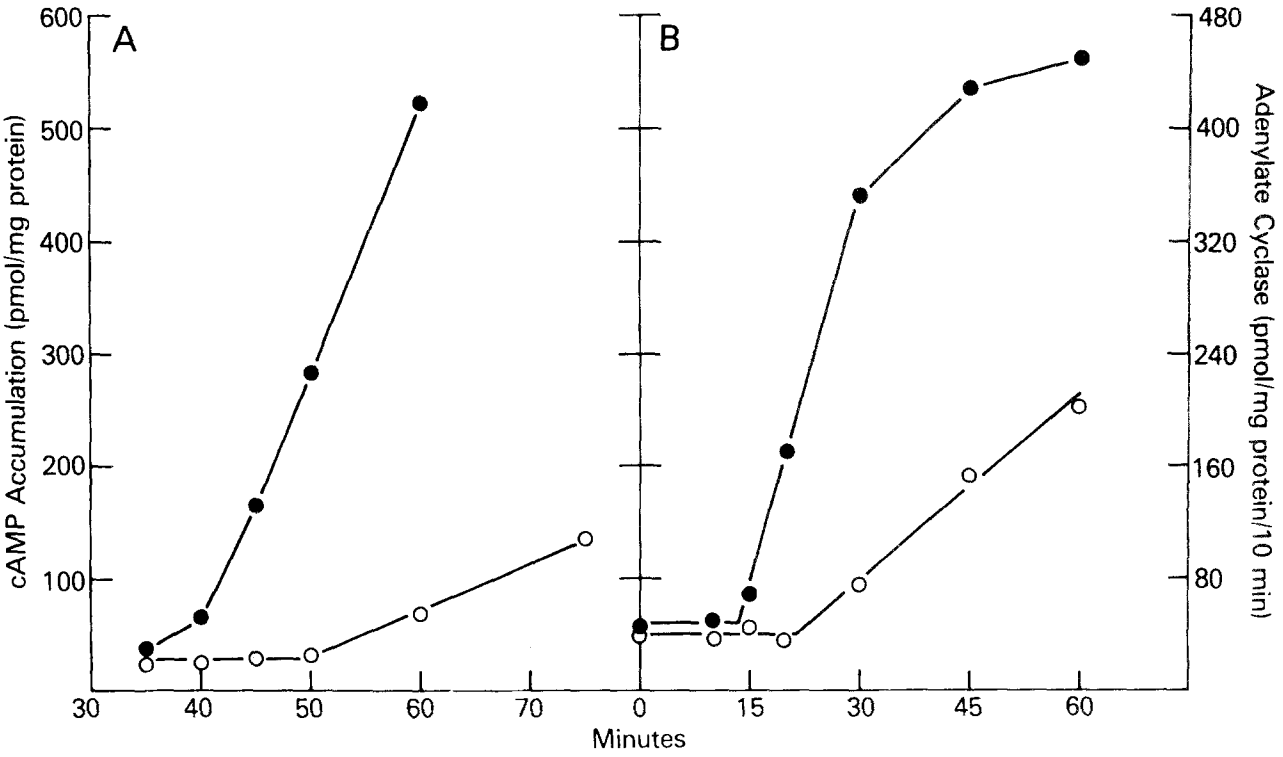


Fig. 3. Lag period for choleragen action on C6 cells treated with a low concentration of G_{M1}. Details are the same as described in the legend to Fig. 2 except as noted. C6 cells were incubated with (•) and without (○) 0.15 μM G_{M1} for 1 hr and washed as described in Materials and Methods. (*A*): Cells were incubated at 37 °C with 10 nM toxin and 0.5 mM MIX and assayed for cAMP content at the indicated times. (*B*): Cells were incubated with 10 nM choleragen at 37 °C and assayed for adenylate cyclase activity at the indicated times

tionally with the concentration of G_{M1} to which the cells were exposed. An enhanced response was observed when the density of toxin receptors was increased < 5-fold. The rate of adenylate cyclase activation by choleragen was half-maximal and maximal when the number of receptors per cell was increased ~10- and ~75-fold, respectively. Under these conditions, each cell bound $\sim 7 \times 10^4$ and 5×10^5 toxin molecules. Even though the number of receptors could be increased further by exposing the cells to more G_{M1}, there was no further increase in adenylate cyclase activity (Fig. 2*C*). Similar results were obtained when cAMP accumulation was measured in cells treated with increasing G_{M1} concentrations. After 50 min exposure to choleragen, the rate of cAMP accumulation was maximal in cells treated with between 0.5 and 1 μM G_{M1} (data not shown).

In addition, cells treated with 0.1 to 0.15 μM G_{M1} still exhibited a shorter lag period than control cells (Fig. 3). Thus, only a 10-fold increase in receptor density is sufficient to cause a significant reduction in the latency period of toxin action as well as an increase in the rate of adenylate cyclase activation and cAMP accumulation.

Effect of Choleragen Concentration

When control and G_{M1}-treated C6 cells were incubated with increasing concentrations of choleragen for 90 min, typical dose response curves for adenylate cyclase activation were obtained (Fig. 4). Half-maximal activation occurred at 2×10^{-10} M for control cells and 2.5×10^{-9} M for G_{M1}-treated cells[2]. Maximal activation occurred at around 10^{-8} M. When the cells were incubated with choleragen for 24 and 48 hr, lower concentrations of toxin were required for activation of adenylate cyclase (Table 2). Half-maximal activation was observed at 1.1×10^{-12} M after 24 hr

[2] The high apparent concentration of choleragen required to activate adenylate cyclase in G_{M1}-treated cells was not unexpected. The treated cells had more receptors than the control cells but were exposed to the same amount to toxin. Thus at 2.5×10^{-9} M choleragen, there were more receptors (10 pmol) than toxin molecules (2.5 pmol) in the incubation; most of the toxin was bound to the cells and the concentration of free toxin was very much lower. In separate experiments with ^{125}I-choleragen under conditions where toxin was in excess, half-saturation of binding sites occurred at 6 and 4×10^{-10} M for control and G_{M1}-treated cells, respectively.

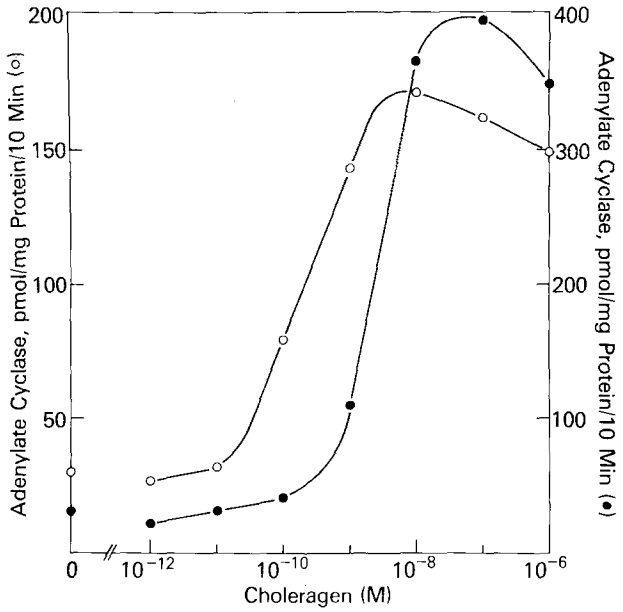


Fig. 4. Effect of choleragen concentration on activation of adenylate cyclase in control and G_{M1}-treated C6 cells. C6 cells cultured in 35 mm wells (1 mg of cell protein per well) were incubated with (●) and without (○) 5 μM G_{M1} for 1 hr, washed, incubated with the indicated concentration of choleragen in 1 ml of medium for 90 min at 37 °C and assayed for adenylate cyclase activity as described in Materials and Methods. In a separate experiment with the cells in suspension, half-maximal activation occurred at 2×10^{-10} and 2.5×10^{-9} M choleragen for control and G_{M1}-treated cells, respectively

Table 2. Effect of choleragen concentration and time of exposure on activation of adenylate cyclase in rat glial C6 cells[a]

Choleragen (M)	Adenylate cyclase activity (pmol/mg protein/10 min) after	
	1 day	2 day
0	55.7	37.0
10^{-14}	–[b]	43.3
10^{-13}	–[b]	81.9[c]
10^{-12}	164[d]	186
10^{-11}	284[e]	241
10^{-10}	284[e]	239

[a] C6 cells cultured in 35 mm wells were incubated at 37 °C with 2 ml of medium containing the indicated concentrations of choleragen and 0.01% bovine serum albumin for 1 or 2 days and assayed for adenylate cyclase activity as described in Materials and Methods. For the 1-day incubation, Medium 199 buffered with 25 mM HEPES was used, and for the 2-day incubation, Dulbecco's minimal essential medium minus serum was used. In both experiments, the cells did not divide but remained viable. After 2 days, some of the cells began to detach from the culture wells, thus precluding conducting such experiments for longer times.

[b] Not determined.

[c] Each well contained 5.0 million cells and each cell was exposed to 24 molecules of toxin.

[d] Each well contained 8.4 million cells and each cell was exposed to 140 molecules of toxin.

[e] Activity in cells treated with 1 μM G_{M1} for 1 hr, washed and incubated for 1 day with 10^{-11} and 10^{-10} M choleragen was 294 and 275 pmol/mg protein/10 min, respectively.

and at 3.5×10^{-13} M after 48 hr[3]. Maximal activation (similar to that observed with G_{M1}-treated cells) required 10^{-11} M choleragen and some activation occured at 10^{-14} M. At this latter concentration, each cell was exposed to ~2 molecules of toxin. Thus, the concentration of choleragen required for half-maximal activation depends on the exposure time and can vary almost 1000-fold.

At lower nonsaturating toxin concentrations, the length of the lag period for both cyclase activation and cAMP accumulation was longer than at saturation (Fig. 5). When C6 cells were treated with low concentrations of G_{M1} and toxin, they bound as much toxin as control cells. Under these conditions the lag period for cAMP accumulation (Fig. 6*A*) and adenylate cyclase activation (Fig. 6*B*) was similar for both control and G_{M1}-treated cells. Thus, the amount of toxin bound per cell appeared to have an effect on the latency period.

[3] At these concentrations, each cell was exposed to 154 and 84 toxin molecules, respectively. If not all of the choleragen was bound and some became inactivated during the long incubation, complete activation of adenylate cyclase may have required only 100 to 200 molecules of toxin bound per cell. In this regard, C6 cells, incubated for 24 hr with 5×10^{-13} M ^{125}I-toxin at 4 or 37 °C, specifically bound 60 and 50%, respectively, of the added toxin.

Effect of Temperature on Choleragen Action

Cells were incubated with choleragen at different temperatures for short periods (up to 90 min), and adenylate cyclase activation and cAMP accumulation were determined (Fig. 7). For both processes, there appeared to be a temperature transition at ~24 °C. Below this temperature, there was little or no cAMP accumulation in G_{M1}-treated cells (Fig. 7*A*) or activation of adenylate cyclase in control (Fig. 7*B*) and G_{M1}-treated cells (not shown). When the cells, however, were incubated for longer times at different temperatures with choleragen, the thermal transition appeared to be at 15 °C (Table 3). At or below 15 °C, cells exposed to toxin for 22 hr did not respond. When these toxin-treated cells were shifted to 37 °C, they did accumulate cAMP, as did cells incubated for 22 hr without choleragen at or below 15 °C and then challenged with toxin at 37 °C (not shown). Thus, the inability of the cells to respond to choleragen at or below 15 °C was not due to an inactivation of the toxin or cells during the long incubation.

Above 15 °C, G_{M1}-treated cells exhibited an enhanced response to choleragen compared to control cells, and higher concentrations of MIX amplified the response of both types of cells (Table 3). The

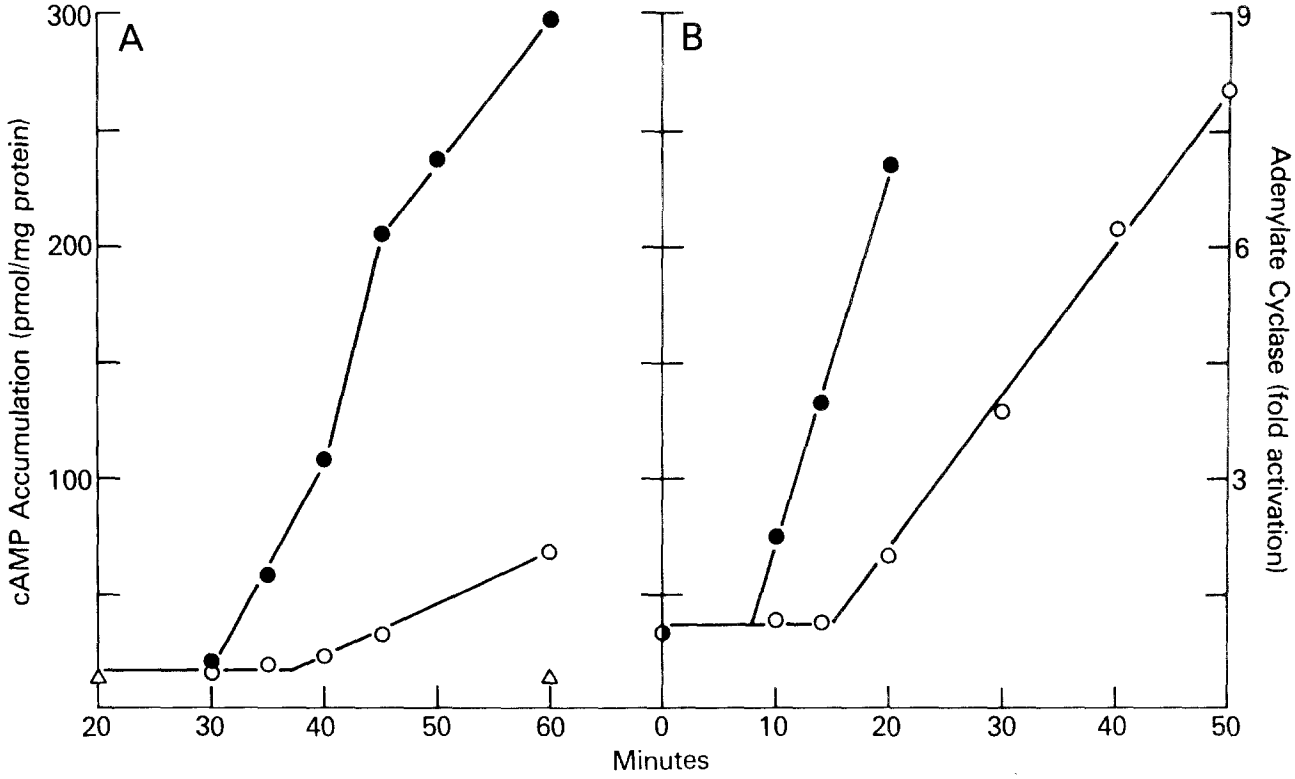


Fig. 5. Effect of choleragen concentration on lag period. (*A*): C6 cells were incubated with 0.5 μM G_{M1} for 1 hr, washed, incubated with 10 nM (●), 1 nM (○), or no (*Δ*) choleragen at 37 °C in medium containing 0.5 mM MIX and assayed for cAMP content at the indicated times as described in Materials and Methods. (*B*): C6 cells treated with 1 μM G_{M1} for 1 hr were washed, suspended in medium containing 20 nM (●) or 1 nM (○) choleragen at 37 °C and assayed for adenylate cyclase activity at the indicated times as described in Materials and Methods

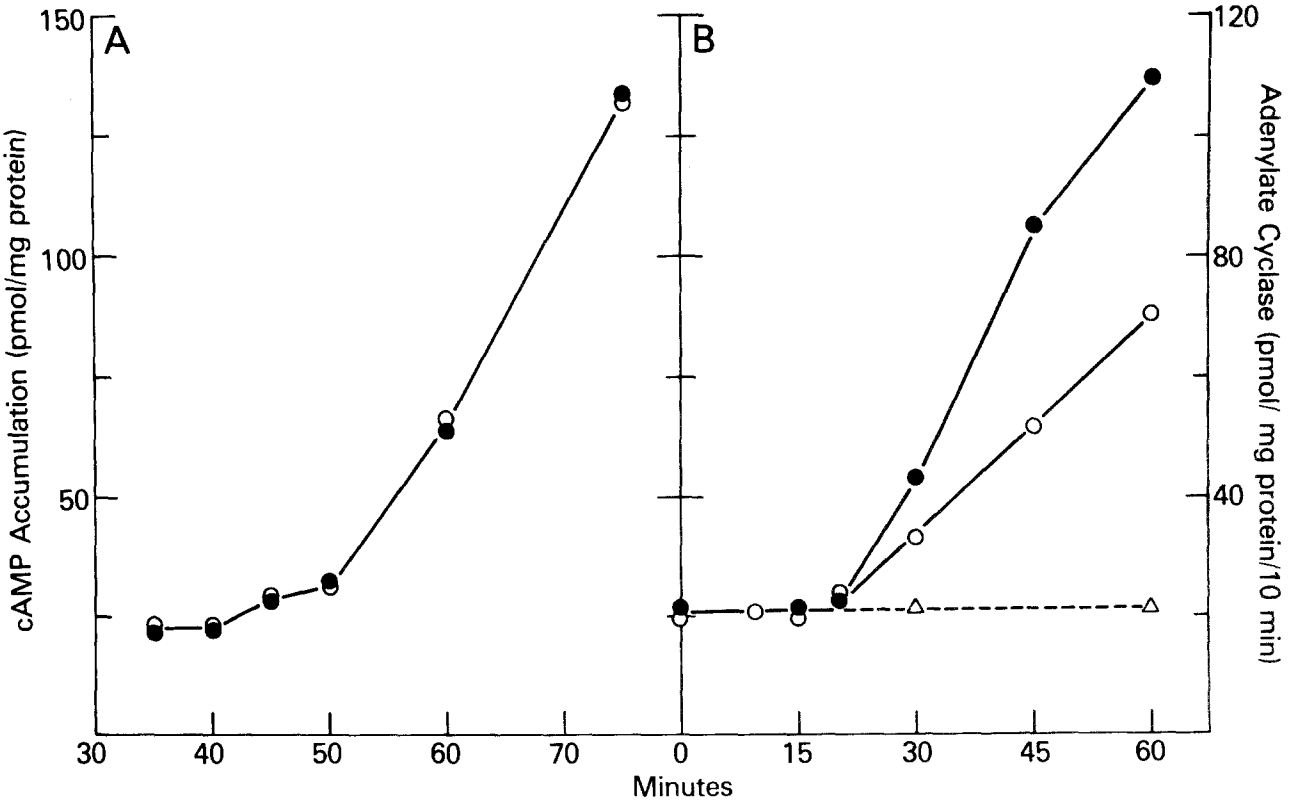


Fig. 6. Effect of bound choleragen on responsiveness of control and G_{M1}-treated C6 cells. (*A*) C6 cells treated with (●) and without (○) 0.15 μM G_{M1} for 1 hr were washed, incubated with 10 (○) or 0.1 (●) nM choleragen plus 0.5 mM MIX at 37 °C, and assayed for cAMP content at the indicated times as described in Materials and Methods. (*B*) – C6 cells treated with (●) and without (○, *Δ*) 0.1 μM G_{M1} for 1 hr were washed, suspended in medium containing 10 nM (●), 0.15 nM (○), or no (*Δ*) choleragen at 37 °C and assayed at the indicated times for adenylate cyclase activity as described in Materials and Methods. Under the conditions described in *A*, control and G_{M1}-treated cells bound 44.4 and 43.9 fmol toxin/mg protein, respectively; under those described in *B*, 45.7 and 88.7 fmol/mg protein, respectively

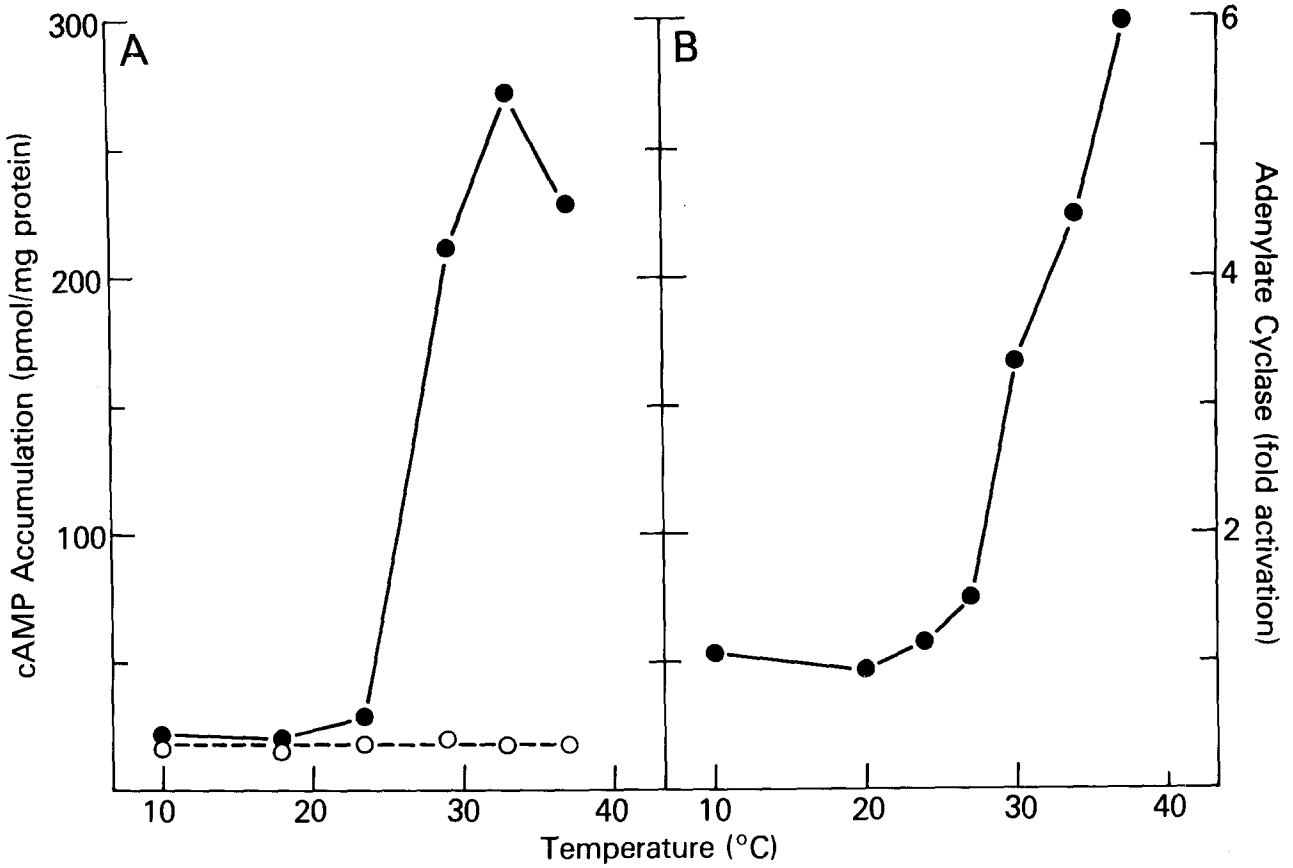


Fig. 7. Effect of temperature on choleragen action. (*A*) C6 cells treated with 0.5 μM G_{M1} for 1 hr were washed and suspended in Medium 199 containing 25 mM HEPES, 0.01% bovine serum albumin and 0.2 mM MIX; 0.5 ml portions were incubated at the indicated temperatures for 90 min with (●) and without (○) 20 nM choleragen, boiled for 5 min, and assayed for cAMP as described in Materials and Methods. (*B*) C6 cells suspended in medium were incubated for 90 min at the indicated temperatures with and without 20 nM choleragen and assayed for adenylate cyclase activity as described in Materials and Methods

Table 3. Effect of temperature on choleragen-stimulated cAMP accumulation in control and G_{M1}-treated rat glial C6 cells[a]

Temperature (°C)	cAMP accumulation (fold stimulation)	
	Control cells	G_{M1}-treated cells
2	0.96	1.11
14	1.27	1.19
15	1.03 (1.10)	1.06 (1.20)
16	1.48	2.93
18	1.80	3.82
20	3.07 (8.79)	8.30 (19.8)

[a] C6 cells cultured in 35 mm wells were incubated with and without 1 μM G_{M1} for 1 hr, washed, and incubated at the indicated temperature in medium with and without 10 nM choleragen plus 0.2 mM MIX; after 22 hr, the cells were assayed for cAMP content as described in Materials and Methods. Values in parentheses represent incubations with 0.5 mM MIX.

time course of cAMP accumulation at 20 and 24 °C is shown in Fig. 8. At both temperatures, the G_{M1}-treated cells exhibited a much shorter lag period than did the control cells. When control cells were incubated for 1 hr with choleragen at 15 °C and then shifted to 25 or 37 °C, there was no significant reduction in the lag period (Fig. 8*C*). Thus, even though toxin can bind to the cells at 15 °C, the event(s) involved in the lag period can not be initiated at this temperature.

G_{M1}-treated C6 cells were incubated with choleragen at 37 °C and then shifted to 15 °C (Fig. 9). No cAMP had accumulated at the time of the temperature transition; cells, however, exposed to toxin for 20 and 30 min at 37 °C began to accumulate cAMP at 15 °C. The rate of acumulation was slower in the cells exposed only 20 min. There was little or no cAMP increase in cells exposed only 10 min to toxin at 37 °C. Similar results were obtained with control C6 cells, except the cells had to be exposed to choleragen for at least 20 min at 37 °C in order to show an increase in cAMP when shifted to 15 °C for 4.5 hr (not shown).

Effects of Antitoxin on Choleragen Action

Addition of anticholeragen antiserum to C6 cells simultaneously with choleragen caused a complete inhi-

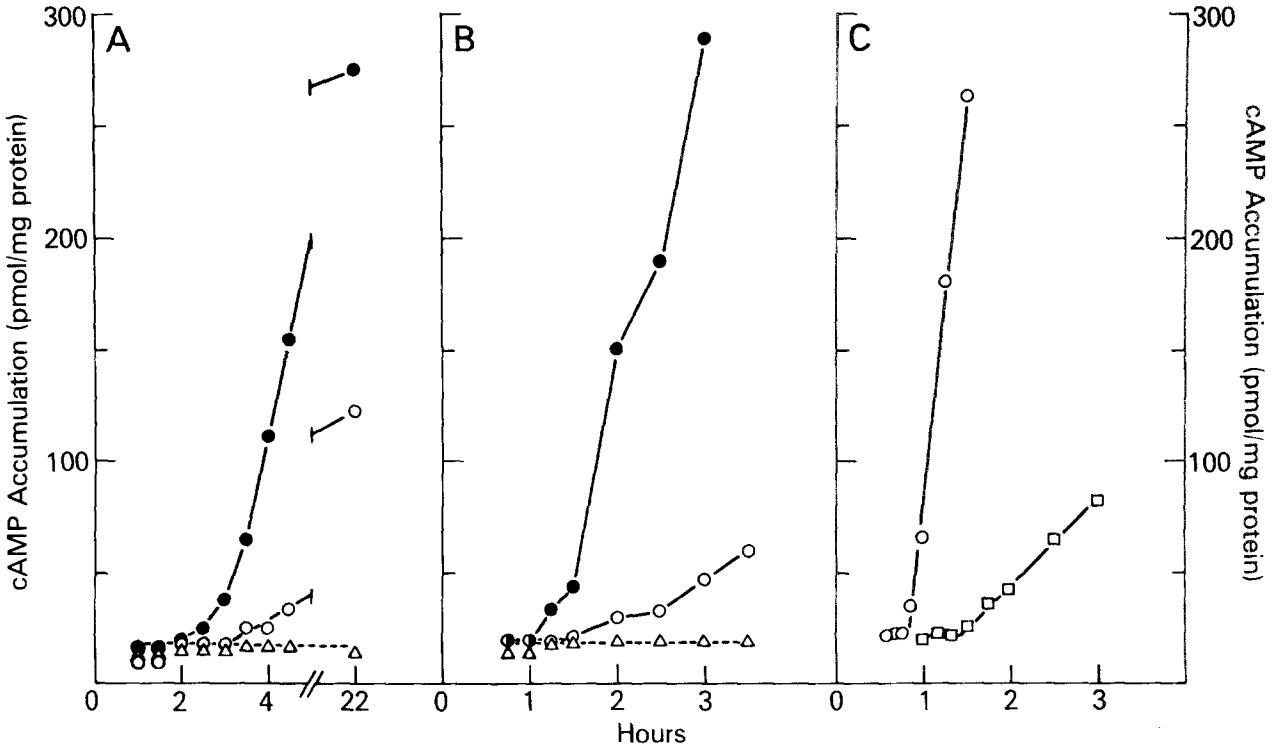
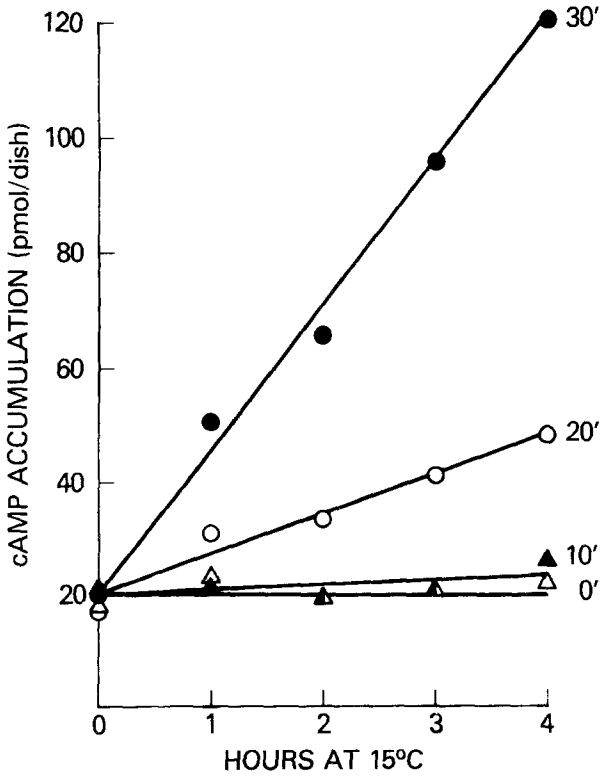


Fig. 8. Effect of temperature on lag period. (*A*) C6 cells treated with (●) and without (○, △) 0.75 μM G_{M1} for 1 hr were washed, incubated with 0.5 mM MIX and 10 nM (●, ○) or no (△) choleragen at 20 °C and assayed for cAMP content at the indicated times as described in Materials and Methods. (*B*) Details the same as in *A* except the cells were incubated at 24 °C. In two additional experiments at 24 °C, the G_{M1}-treated cells began to accumulate cAMP after 60 min and the control cells after 90 min. (*C*) C6 cells were incubated in 1 ml of medium with 10 nM choleragen for 1 hr at 15 °C and 1 ml of warm medium with 1 mM MIX was added; the cells were shifted to 37 °C (○) or 25 °C (□) and assayed for cAMP content at the indicated times



bition of adenylate cyclase activation (Fig. 10*A*). Addition of the antitoxin even 20 sec after the toxin to G_{M1}-treated cells at 37 °C resulted in significant activation of adenylate cyclase. When added after 1 min, the antitoxin was only 50% effective. In contrast, activation of adenylate cyclase in control C6 cells was completely blocked even when the antitoxin was added after 1 min; the antitoxin was 50% effective even when added 6 min after the toxin. Under these latter conditions, adenylate cyclase activity increased with time but at a slower rate than in cells not exposed to antitoxin (not shown). The cells were then incubated at different temperatures with choleragen for 15 or 20 min, exposed to antitoxin, and incubated further at 37 °C (Fig. 10*B*). At or below 15 °C, the antitoxin was completely effective in blocking acti-

Fig. 9. Effect of exposure time at 37 °C on choleragen-stimulated cAMP production in G_{M1}-treated C6 cells incubated at 15 °C. C6 cells were treated with 0.75 μM G_{M1} for 1 hr, washed and incubated at 37 °C with 10 nM choleragen in 1 ml of medium for 0 (△), 10 (▲), 20 (○), or 30 (●) min; then 1 ml of ice-cold medium containing 1 mM MIX was added and the cells were incubated at 15 °C and assayed for cAMP content at the indicated times as described in Materials and Methods. The cAMP content of cells incubated at 15 °C without toxin for up to 4 hr varied between 19.3 and 23.6 pmol/dish

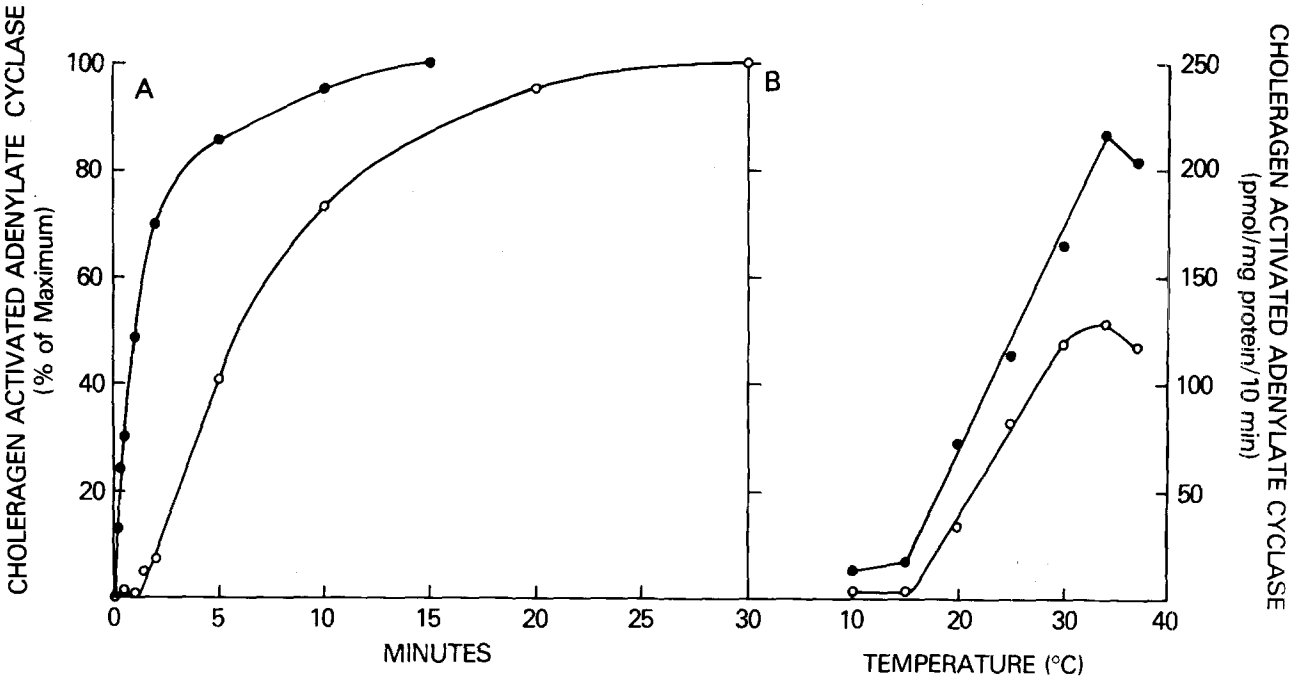


Fig. 10. Effect of antitoxin on activation of adenylate cyclase by choleragen. (*A*) C6 cells were treated with (●) and without (○) 5 μM G_{M1} for 1 hr, washed and suspended in Medium 199 containing 25 mM HEPES and 0.01% bovine serum albumin; 1 ml portions were incubated at 34 °C with 10 nM choleragen, and antitoxin (10 μl) was added at the indicated times; after 1 (●) or 1.5 (○) hr, the cells were assayed for adenylate cyclase activity as described in Materials and Methods (*B*): Details are the same as in *A* except the cells were incubated with choleragen at the indicated temperatures for 10 (●) or 20 (○) min prior to adding the antitoxin; then the cells were shifted to 37 °C, incubated an additional 80 (○) or 50 (●) min, and assayed for adenylate cyclase activity

Table 4. Effects of antitoxin on binding of ^{125}I-choleragen to rat glial C6 cells[a]

Conditions	^{125}I-toxin bound (fmol/mg protein)	
	Control cells	G_{M1}-treated cells
1 hr at 37 °C	38.2	6485
Antitoxin, then 1 hr at 37 °C	0	0
20 min at 37 °C, then antitoxin and 40 min at 37 °C	24.2	3349
20 min at 4°C, then antitoxin and 40 min at 37 °C	19.6	1224

[a] C6 cells were treated with and without 5 μM G_{M1} for 1 hr, washed, and suspended in medium. Portions were incubated as indicated in 0.2 ml of Tris-buffered saline (pH 7.4) containing 0.1% bovine serum albumin and 5×10^{-10} M ^{125}I-choleragen. When added, the amount of antitoxin was 2 μl. The samples were filtered and analyzed for bound iodotoxin as described in Materials and Methods. The incubations contained sufficiently few cells that less than 25% of the iodotoxin was bound. Values have been corrected for nonspecific binding as measured in the presence of 2.4×10^{-7} M unlabeled choleragen.

vation of adenylate cyclase in both types of cells. Above 15 °C, inhibition of the activation process decreased with increasing temperature.

In separate experiments with ^{125}I-choleragen, the antitoxin, when added first, blocked the binding of the labeled toxin but, when added after the toxin, could only displace part of it (Table 4). Although displacement of toxin was greater from cells preincubated at 4 than at 37 °C for 20 min, sufficient toxin remained bound in the presence of the antitoxin at 4 °C to theoretically activate adenylate cyclase. Thus, the ability of the antitoxin to block activation of adenylate cyclase by bound toxin at or below 15 °C does not depend on its ability to displace the toxin.

Discussion

The number of choleragen receptors per cell has profound effects on toxin responsiveness. Mouse neuroblastoma N18 cells and G_{M1}-treated rat glial C6 cells responded more rapidly than control C6 cells and exhibited a significantly shorter lag period[4]. Even a 10-fold increase in toxin receptors resulted in a reduction (Fig. 3). Others also have reported that treatment of cells with G_{M1} enhanced their sensitivity to choleragen; these included rat adipocytes [6, 30], pigeon erythrocytes [14, 24], rabbit intestinal mucosa

[4] HeLa cells have few choleragen receptors [8, 9], which can be increased by treating the cells with G_{M1} [9] or culturing them with sodium butyrate [8]. With either treatment, the HeLa cells exhibited a much shorter lag period than control cells when exposed to choleragen (P.H. Fishman and R.C. Henneberry, *unpublished*).

[18], and G_{M1}-deficient mouse fibroblasts [11, 26]. Effects of receptor density, however, have not been noted on the lag period for the activation of adenylate cyclase by choleragen.

As expected, choleragen responsiveness was modulated by its concentration. The effects of toxin concentration, however, were highly dependent on exposure time (Fig. 4 and Table 2). Thus, half-maximal activation of adenylate cyclase, when measured at 1.5, 24, and 48 hr, required 200, 1.1, and 0.35 pM choleragen, respectively. These results are consistent with a catalytic model of choleragen action as first suggested by Gill [12–14], in which a few toxin molecules bound to the cell progressively activate more and more adenylate cyclase. Such a mechanism has been demonstrated in disrupted cells with A_1 peptide [12] but not previously in intact cells. In contrast, Cuatrecasas and coworkers proposed that the activation process involved a bimolecular interaction between the toxin and adenylate cyclase, each toxin molecule activating one cyclase [1, 2, 30]. With a variety of different cell types, they observed that maximal activation occurred when each cell bound 1 to 2×10^4 toxin molecules [2, 30]. In the studies reported here, maximal activation can occur in 30 min with 5×10^5 molecules bound per cell or in 48 hr with 100–200 molecules bound per cell.

Choleragen concentration also influenced the lag period. When G_{M1}-treated cells were exposed to subsaturating levels of toxin, the latency period increased (Fig. 5). When conditions were adjusted so that similar amounts of toxin were bound to control and G_{M1}-treated cells, the lag periods were essentially the same (Fig. 6). Thus, the decrease in latency observed with G_{M1}-treated cells may represent in part the increased amount of choleragen that binds to them.

Bennett and Cuatrecasas reported that rat adipocytes displayed a temperature transition at 25 °C for activation of adenylate cyclase by choleragen [2]. A similar transition was observed with rat glial C6 cells incubated for short times; but with prolonged exposure to toxin, the transition was at 15 °C and appeared to be absolute. Even after 22 hr, no stimulation by choleragen was observed at or below this temperature in either control or G_{M1}-treated cells. In addition, preincubation at 15 °C with choleragen did not cause any significant reduction in the lag period when the cells were shifted to a higher temperature. The inertness of bound choleragen at or below 15 °C further evident by the experiments with antitoxin, which completely blocked the activation of cyclase in cells preincubated with choleragen at or below this temperature. An effect of temperature on the lag period also was observed. The higher the temperature, the shorter the latency period; and at each temperature, G_{M1}-treated cells always exhibited a shorter lag than control cells.

Once choleragen activated adenylate cyclase, the cells accumulated cAMP when shifted to or below 15 °C. Gill reported that the A_1 subunit activated adenylate cyclase in disrupted cells even below 15 °C [12]. Thus, this thermal barrier in intact cells is consitent with the inability of choleragen bound to the cell surface to gain access to adenylate cyclase. In this regard, the intact cells responded at 15 °C only when exposed at 37 °C to choleragen for >10 min (G_{M1}-treated) or >20 min (control). These are the minimum lag times for adenylate cyclase activation at 37 °C (Fig. 2).

From the experiments with antitoxin, it appears that at 37 °C choleragen rapidly binds to the cell surface and becomes "inaccessible" to inhibition by the antitoxin. Gill and King also reported a rapid "eclipse" of inhibition by antitoxin in pigeon erythrocytes [14]. Yet even under optimal conditions, the cell-bound toxin, though "inaccessible" to inhibition by antitoxin, is unable to activate adenylate cyclase for several minutes. In disrupted cells, activation by A_1 peptide can occur immediately [12], and further activation can be be inhibited by antitoxin [14]. The sharp temperature transition for the activation of adenylate cyclase and for the inhibition of this activation by antitoxin and by G_{M1}-oligosaccharide [9] implies that the bound toxin forms an "irreversible" association with the cell membrane above this temperature.

This association presumably involves the penetration of choleragen or some part of it into the membrane. Because of the tight, essential irreversible binding of the B component to the surface receptors with increasing time and temperature [7, 17], the A component only may initially penetrate into the membrane. The lag period would then represent the time required for the A component to penetrate across the lipid bilayer and become reduced to A_1 peptide, which can catalyze the activation of adenylate cyclase. This last process involves the NAD-dependent ADP-ribosylation of a GTP-binding protein that regulates cyclase activity [4, 15, 20]. Thus, even a few A_1 molecules can eventually activate all of the cyclase in a cell. The more A_1 present, the more rapidly the activation process occurs.

This model is consistent with the topological orientation of the toxin receptors and the adenylate cyclase complexes on the external and internal halves of the plasma membrane. Whether only the A component or the holotoxin initially penetrates across the membrane is not yet known. Numerous experimental approaches have been attempted with am-

biguous results. Hansson et al. [16], using an immunochemical technique, reported that choleragen remained at the cell surface below 18 °C, but after 30 min at 37 °C some of the toxin had penetrated into the membrane. The antitoxin used in these studies reacted with both the A and B components. Using a histochemical procedure, Joseph et al. [21] observed that endocytosis of choleragen, as well as the B component conjugated to horseradish peroxidase, occurred within 30 min at 37 °C but not at 4 °C. The internalized material accumulated in structures of the cell referred to as GERL (Golgi apparatus-endoplasmic reticulum-lysosomes). Whether either of the two molecules of peroxidase per toxin were conjugated to the A component was not determined. Also, it was not clear what effect these peroxidases, which are glycoprotein and which double the effective size of the toxin, had on the results. Both of these studies indicate that internalization occurs, but it may be subsequent to the activation process and may involve the eventual degradation of choleragen[5].

Attempts to detect any ^{125}I-choleragen inside the cells following brief exposure to the toxin at 37 °C have been unsuccessful [2, 17]. These experiments would not discriminate between iodotoxin bound to the external surface of the plasma membrane or within the membrane. More recently, evidence has been presented for the penetration of the A_1 but not the B peptides into the membrane bilayer [35]. The technique involved the incorporation into the membrane of an artificial lipid containing a photoreactive group positioned 13 Å from the membrane surface. When choleragen was incubated with the membranes for 15 min at 37 °C but not 0 °C, the A_1 peptide became labeled after irradiation of the membranes. Although these results are strongly supportive of the model presented here, failure to label the B component of the toxin may represent some technical problem. Additional experiments will be necessary to clarify which portion(s) of choleragen penetrate across the membrane during the lag phase.

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[5] Recently, the degradation of ^{125}I-choleragen bound to normal human fibroblasts has been followed [27]. The iodotoxin appears to have a long half-life (24 hr). With loss of cell-associated radioactivity, there is a corresponding appearence of low molecular weight radioactivity in the culture medium. Most of the cell-associated radioactivity could be precipitated with trichloroacetic acid and corresponded to the A_1 and B peptides when analyzed by SDS-polyacrylamide gel electrophoresis. Thus, the iodotoxin appears to be slowly degraded, presumably in the lysosomes, with excretion of iodotyrosine into the culture medium.

References

1. Bennett, V., Craig, S., Hollenberg, M.D., O'Keefe, E., Sahyoun, N., Cuatrecasas, P. 1976. Structure and function of cholera toxin and hormone receptors. *J. Supramol. Struct.* **4**:99
2. Bennett, V., Cuatrecasas, P. 1975. Mechanism of activation of adenylate cyclase by *Vibrio cholerae* enterotoxin. *J. Membrane Biol.* **22**:29
3. Brady, R.O., Fishman, P.H. 1979. Biotransducers of membrane-mediated information. *Adv. Enzymol.* **50**:303
4. Cassel, D., Pfeuffer, T. 1978. Mechanism of cholera toxin action: Covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Nat. Acad. Sci. USA* **75**:2669
5. Cuatrecasas, P. 1973*a*. Interaction of *Vibrio Cholerae* enterotoxin with cell membranes. *Biochemistry* **18**:3547
6. Cuatrecasas, P. 1973*b*. Gangliosides and membrane receptors for cholera toxin. *Biochemistry* **18**:3558
7. Cuatrecasas, P. 1973*c*. *Vibrio cholerae* choleragenoid. Mechanism of inhibition of cholera toxin action. *Biochemistry* **18**:3577
8. Fishman, P.H., Atikkan, E.E. 1979*a*. Induction of cholera toxin receptors in cultured cells by butyric acid. *J. Biol. Chem.* **254**:4342
9. Fishman, P.H., Atikkan, E.E. 1979*b*. Mechanism of action of cholera toxin: Effect of receptor density and multivalent binding on activation of adenylate cyclase. *J. Membrane Biol.* **54**:51
10. Fishman, P.H., Moss, J., Richards, R.L., Brady, R.O., Alving, C.R. 1979. Liposomes as model membranes for ligand-receptor interactions: studies with choleragen and glycolipids. *Biochemistry* **18**:2562
11. Fishman, P.H., Moss, J., Vaughan, M. 1976. Uptake and metabolism of gangliosides in transformed mouse fibroblasts. Relationship of ganglioside structure to choleragen response. *J. Biol. Chem.* **251**:4490
12. Gill, D.M. 1976. Multiple roles of erythrocyte supernatant in the activation of adenylate cyclase by *Vibrio cholerae* toxin *in vitro*. *J. Infect. Dis.* **133**:S55
13. Gill, D.M. 1977. The mechanism of action of cholera toxin. *Adv. Cyclic Nucleotide Res.* **8**:85
14. Gill, D.M., King, C.A. 1975. The mechanism of action of cholera toxin in pigeon erythrocyte lysates. *J. Biol. Chem.* **250**:6424
15 Gill, D.M., Meren, R. 1978. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: Basis of the activation of adenylate cyclase. *Proc. Nat. Acad. Sci. USA* **75**:3050
16. Hansson, H.-A, Holmgren, J., Svennerholm, L. 1977. Ultrastructural localization of cell membrane G_{M1} ganglioside by cholera toxin. *Proc. Nat. Acad. Sci. USA* **74**:3782
17. Homgren, J., Lonnroth, I. 1976. Cholera toxin and the adenylate cyclase-activating signal. *J. Infect. Dis.* **133**:S64
18. Holmgren, J., Lonnroth, I., Mansson, J.-E., Svennerholm, L. 1975. Interaction of cholera toxin and membrane G_{M1} ganglioside of small intestine. *Proc. Nat. Acad. Sci. USA* **72**:2520
19. Holmgren, J., Lonnroth, I., Svennerholm, L. 1973. Tissue receptor for cholera exotoxin: Postulated structure from studies with G_{M1} ganglioside and related glycolipids. *Infect. Immun.* **8**:208
20. Johnson, G.L., Kaslow, H.R., Bourne, H.R. 1978. Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. *J. Biol. Chem.* **253**:7120
21. Joseph, K.C., Stieber, A., Gonatas, N.K. 1979. Endocytosis of cholera toxin in GERL-like structures of murine neuroblastoma cells pretreated with G_{M1} ganglioside. *J. Cell Biol.* **81**:543
22. Kimberg, D.V., Field, M., Johnson, J., Henderson, A., Gershon, E. 1971. Stimulation of intestinal mucosyl adenylate

cyclase by cholera enterotoxin and prostaglandin. *J. Clin. Invest.* **52**:1376
23. King, C.A, van Heyningen, W.E. 1973. Deactivation of cholera toxin by a sialidase-resistant monosialoganglioside. *J. Infect. Dis.* **127**:639
24. King, C.A., van Heyningen, W.E., Gascoyne, N. 1976. Aspects of the interaction of *Vibrio cholerae* toxin with the pigeon red cell membrane. *J. Infect. Dis.* **133**:S75
25. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265
26. Moss, J., Fishman, P.H., Manganiello, V.C., Vaughan, M., Brady, R.O. 1976. Functional incorporation of ganglioside into intact cells: induction of choleragen responsiveness. *Proc. Nat. Acad. Sci. USA* **73**:1034
27. Moss, J., Fishman, P.H., Watkins, P.A. 1979. *In vivo* degradation of ^{125}I-choleragen by normal human fibroblasts. *In:* Fifteenth Joint Conference on Cholera and Related Diarrheal Diseases, United States-Japan Cooperative Medical Science Program. N. Pierce and K. Takeya, editors. (*in press*)
28. Moss, J., Manganiello, V.C., Fishman, P.H. 1977. Enzymatic and chemical oxidation of gangliosides in cultured cells: Effects of choleragen. *Biochemistry* **16**:1876
29. Moss, J., Vaughan, M. 1977. Mechanism of action of choleragen. Evidence for ADP-ribosyltransferase activity with arginine as an acceptor. *J. Biol. Chem.* **252**:2455
30. O'Keefe, E., Cuatrecasas, P. 1978. Cholera toxin and membrane gangliosides: Binding and adenylate cyclase in normal and transformed cells. *J. Membrane Biol.* **42**:61
31. Pacuszka, T., Duffard, R.O., Nishimura, R.N., Brady, R.O., Fishman, P.H. 1978. Biosynthesis of bovine thyroid gangliosides. *J. Biol. Chem.* **253**:5839
32. Svennerholm, L. 1963. Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10**:613
33. Terasaki, W.L., Brooker, G. 1978. [^{125}I]iodohydroxybenzylpindolol binding sites on intact rat glioma cells. Evidence for β-adrenergic receptors of high coupling efficiency. *J. Biol. Chem.* **253**:5418
34. Van Heyningen, S. 1977. Cholera toxin. *Biol. Rev.* **52**:509
35. Wisnieski, B.J., Bramhall, J.S. 1979. Labeling of of the active subunit of cholera toxin from within the membrane bilayer. *Biochem. Biophys. Res. Commun.* **87**:308

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