Biochem. J. (1990) 271, 107–111 (Printed in Great Britain)

Cyclic AMP accumulation in HeLa cells induced by cholera toxin

Involvement of the ceramide moiety of GM1 ganglioside

Massimo MASSERINI,*‡ Paola PALESTINI,* Marina PITTO,* Vanna CHIGORNO,* Maurizio TOMASI† and Guido TETTAMANTI*

*Study Center for the Functional Biochemistry of Brain Lipids, Department of Medical Chemistry and Biochemistry, The Medical School, University of Milan, 20133 Milano, and †Istituto Superiore di Sanità, Roma, Italy

The influence of ceramide composition on the rate of GM1 association to HeLa cells has been investigated by incubating the cells in the presence of either native ganglioside or molecular species carrying highly homogeneous long chain base moieties, fractionated from native GM1. The GM1 ganglioside species carrying the unsaturated C_{18} long chain base moiety proved to have the fastest rate of association, whereas the saturated species carrying 20 carbon atoms had the slowest rate. After having increased the GM1 cell content (65-fold) by incubation with the various ganglioside species, the cells were incubated with cholera toxin and the time course of cyclic AMP accumulation was monitored. Remarkable differences among cells enriched with the various molecular species were found in the duration of the lag time preceding the accumulation of cyclic AMP, the shortest being displayed by the unsaturated C_{18} species. Moreover, the amount of cyclic AMP accumulated after a given time of incubation with cholera toxin was significantly higher when the $C_{18:1}$-GM1 species was present than with native GM1. Fluorescence anisotropy experiments, carried out using the probe 1,3-diphenylhexatriene, show that the GM1 ganglioside ceramide moiety was also modifying the cell membrane fluidity of the host.

INTRODUCTION

Cholera toxin (CT) is composed of two subunits, A and B, the latter being in a pentameric form. Subunit A consists of two peptides, A1 and A2, linked by a disulphide bond (van Heyningen, 1983). When CT interacts with the host cells it triggers a response which results in the irreversible activation of adenylate cyclase (Holmgren, 1981; Fishman, 1982; Van Heyningen, 1983). Firstly, the CT binds through the B subunits to its receptor, GM1 ganglioside. A lag period then follows during which the A subunit penetrates the plasma membrane and splits into peptides A1 and A2; the A1 fragment catalyses the NAD^+-dependent ADP-ribosylation of the regulatory guanine-nucleotide-binding (G-protein), which irreversibly binds to, and activates, adenylate cyclase.

GM1 ganglioside is an amphiphilic molecule containing an oligosaccharide hydrophilic portion and a lipid moiety (ceramide), composed of a sphingoid long chain base (LCB) and a fatty acid linked through an amide bond. It has been definitely established that the binding of CT to GM1 involves the oligosaccharide portion of the glycolipid (Fishman *et al.*, 1978), whereas the involvement of the lipid portion is virtually unknown (Wiegandt *et al.*, 1976; Fishman *et al.*, 1980). GM1 ganglioside, as found in cells ('native GM1') has a heterogeneous lipid portion (Sonnino *et al.*, 1984). In particular, the composition is characterized by a prevalence of C_{18} and C_{20} LCB unsaturated species, and to a lesser extent of the corresponding saturated species. The aim of the present investigation was to study the effects of differences in the lipid portion of GM1 on the binding and activatory action of CT on adenylate cyclase. Our experimental approach was based on the observation that cells lacking the receptor can be rendered susceptible to the toxin by incorporation of exogenous GM1 ganglioside (Moss *et al.*, 1976). Therefore we decided to enrich cultured HeLa cells, known to possess a very low GM1 content (Fishman & Atikkan, 1979; Markwell *et al.*, 1984), with GM1 molecular species carrying each of the LCBs present in the native ganglioside, and to study the responsiveness of the cells to CT by measuring the modifications in the cellular cyclic AMP content.

MATERIALS AND METHODS

Reagents and other products

Commercial chemicals were of the purest quality available; solvents were distilled and water doubly distilled in a glass apparatus. Silica-gel-precoated thin layer plates (Kieselgel 60; 20 cm × 20 cm aluminium sheets) were from Merck (Darmstadt, Germany); trypsin, *N*-acetylneuraminic acid and crystalline BSA were from Sigma (St. Louis, MO, U.S.A.). All solutions for HeLa cell culture and washing were from Flow Laboratories (Irvine, Scotland, U.K.). CT was purified as described by Tomasi *et al.* (1979), using the phosphocellulose chromatography modifications introduced by Mekalanos *et al.* (1979).

Ganglioside preparation

Ganglioside GM1 was prepared from calf brain according to Tettamanti *et al.* (1973) and was ^{3}H-labelled at the terminal galactose moiety by the method of Suzuki & Suzuki (1972), as modified by Ghidoni *et al.* (1974). The specific radioactivity was 1.08 Ci/mmol and the radioactivity purity greater than 99%. The separation of native GM1, radiolabelled or not, into the molecular species with homogeneous LCB composition was attained using reverse-phase h.p.l.c. as described by Sonnino *et al.* (1984), with the modifications introduced by Masserini & Freire (1986). The saturated species were prepared by hydro-

Abbreviations used: EMEM, Eagle's minimum essential medium with Earle's salts; PBS, Dulbecco's phosphate-buffered saline solution without calcium and magnesium; FCS, fetal calf serum; CT, cholera toxin; h.p.t.l.c., high performance thin layer chromatography; LCB, long chain base; DPH, 1,6-diphenylhexa-1,3,5-triene; G-protein, regulatory GTP-binding protein.

‡ To whom correspondence should be addressed at: Department of Biochemistry, Università Milano, Via Saldini 50, 20133 Milano, Italy.

genation of the corresponding unsaturated species according to Sonnino *et al.* (1984). By these procedures GM1 ganglioside species containing the C_{18} or C_{20} LCB in the unsaturated ($C_{18:1}$, $C_{20:1}$) or saturated ($C_{18:0}$, $C_{20:0}$) form were prepared. Identification, structural analysis and sample purity were assessed as described by Sonnino *et al.* (1978, 1984). The final purity of all gangliosides was greater than 99% with regard to the oligosaccharide portion. For molecular species it was greater than 99% also with regard to the LCB composition. The LCB composition of native GM1 was: $C_{18:1}$, 56% $C_{20:1}$, 37%; $C_{18:0}$, 4%; $C_{20:0}$, 3%. The fatty acid composition of all gangliosides showed that they contained more than 93% stearic acid.

Cell culture conditions

HeLa cells were propagated in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) as described by Henneberry (1975). Subcultures were made on 6 cm (diameter) culture dishes using 5 ml of EMEM containing 10% (v/v) FCS. Cells used for experiments were at 80–90% confluency (300–350 μg of cell protein/dish; about 9×10^5 cells). Harvesting, rupture and homogenization of cells were carried out as described by Chigorno *et al.* (1986). In particular, the 'light' membrane subfraction (plasma-membrane enriched), was prepared and characterized as described by Chigorno *et al.* (1986). Cell viability was assessed by the Trypan Blue absorption method (Phillips, 1973). The morphology of the cultured cells and the efficiency of the homogenization procedure were examined using a phase-contrast microscope.

Treatment of HeLa cells with GM1

[^{3}H]GM1-containing medium (2 ml; without FCS; about 10^6 d.p.m./ml) was added to each dish and incubated at 37 °C for up to 24 h. At the end of incubation the cell monolayers were washed three times with 3 ml (each time) of 0.9% aq. NaCl in order to remove unbound GM1 (1000 *g*, 10 min) and then maintained at 37 °C for 30 min with 2 ml of EMEM containing 10% FCS to remove the GM1 molecules which were weakly adhering to the cells. The cell preparations obtained after FCS treatment contained the 'serum-stable' fraction of associated ganglioside (Schwarzmann *et al.*, 1983; Chigorno *et al.*, 1985). In some experiments cells were further treated (5 min at 37 °C) with 2 ml of phosphate buffer containing 0.1% trypsin in order to remove GM1 interacting with trypsin-sensitive membrane proteins (Schwarzmann *et al.*, 1983; Chigorno *et al.*, 1985). The cell preparations obtained after trypsin treatment contained the 'trypsin-stable' fraction of associated ganglioside. At the end of the treatment, cells were harvested by scraping with a rubber policeman and were pelleted by centrifugation (1000 *g*, 10 min).

Assessment of the amount of GM1 associated with cells

Gangliosides were extracted from cells containing the serum-stable or trypsin-stable form, according to Tettamanti *et al.* (1973). Volatile radioactivity and lipid-bound radioactivity were determined as described by Trinchera *et al.* (1988). Individual gangliosides were separated by high performance thin layer chromatography (h.p.t.l.c.). The radioactivity was counted after overnight treatment of an aliquot of cells with 1 M-NaOH (3 ml/mg of cell protein).

T.l.c. of gangliosides

Plates were developed at room temperature with the solvent system chloroform/methanol/0.2% $CaCl_2$ (50: 42:11, by vol.) Ganglioside spots were made visible by autoradiography and quantified by radiochromatoscanning of the plate or by densitometry (Ghidoni *et al.*, 1986).

Treatment of cells with CT and assay of cyclic AMP

An established amount of CT in 2 ml of EMEM (without FCS) was added to the cells. The EMEM solution contained BSA (0.01%) and 3-isobutyl-1-methylxanthine (0.2 mM) as the phosphodiesterase inhibitor (Fishman *et al.*, 1980). After a period of incubation, cyclic AMP was extracted and assayed. For this purpose, the cells were treated with 1.5 ml of an ice-cold trichloroacetic acid solution (6%, w/v), scraped off and centrifuged at 10000 *g* (15 min). The pellet obtained was utilized for protein assay and the supernatant for cyclic AMP assay. For this purpose, the supernatant was extracted (four times) with 5 vol. of diethyl ether saturated with water, the aqueous solution was lyophilized, and the residue was resuspended in water and assayed for cyclic AMP by radioimmunoassay (RPA509 cyclic AMP assay system; Amersham, Little Chalfont, Bucks., U.K.).

Colorimetric methods

Protein content was determined in solubilized cell pellets, according to Peterson (1977), with BSA as the reference standard. Lipid-bound sialic acid was determined according to Svennerholm (1957).

Fluorescence measurements

The steady-state degree of fluorescence polarization (*P*) was calculated for DPH (1,6-diphenylhexa-1,3,5-triene) incorporated into the light membrane subfractions prepared from cells enriched with native GM1, $C_{18:1}$-GM1 or $C_{20:0}$-GM1.

The fluorophore was added to the cell membrane preparation from acetonitrile solutions (Shinitzky & Barenholz, 1978) and equilibrated after incubation under continuous stirring for 40 min at 30 °C. Determination was carried out by simultaneously measuring the intensities of parallel and perpendicularly polarized light (Shinitzky & Barenholz, 1978) with a Jasco spectrofluorimeter (Jasco, Tokyo, Japan).

RESULTS

Binding experiments

The time course of native GM1 association (serum-stable form) to HeLa cells, following incubation with ganglioside at concentrations of 1, 5, 10 and 50 μM, is reported in Fig. 1(*a*). The time course of association of GM1 molecular species with a homogeneous LCB composition was monitored by incubating the cells in the presence of the highest glycolipid concentration (50 μM), leading to the association of larger ganglioside amounts. Results are reported in Fig. 1(*b*). As shown, the rate of association to cells was faster for $C_{18:1}$-GM1 than for the other molecular species, the slowest rate being recorded for $C_{20:0}$-GM1. The rates of association of native GM1 and of the other molecular species were between these values. The content of endogenous GM1 ganglioside in cells was 20 pmol/mg of protein.

Cell viability after incubation with native GM1 or with GM1 molecular species was good for up to 24 h.

In some instances (cells treated with native GM1, $C_{18:1}$-GM1 and $C_{20:0}$-GM1) the cells were further treated with trypsin: results showed that the amount of the trypsin-stable form of gangliosides associated to cells was 59%, 55% and 45% of the serum-stable form for native GM1, $C_{18:1}$-GM1 and $C_{20:0}$-GM1 respectively. After trypsin treatment, the cells were generally found to become non-viable and tended to detach from the dish.

On the basis of the above experiments, different incubation times were chosen to bring about an association to the cells of identical amounts of ganglioside molecular species (serum-stable form of association). The times chosen, leading to the association of 1.3 ± 0.1 nmol/mg of protein, were 80 min for $C_{18:1}$-GM1,

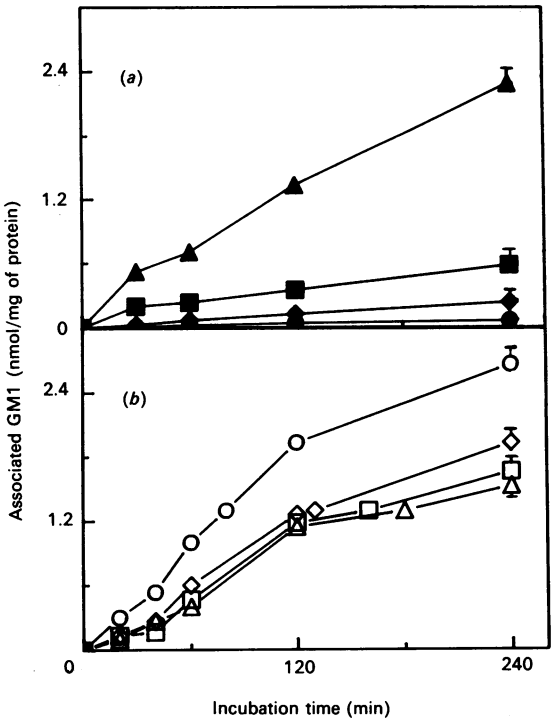

Fig. 1. Time course of GM1 ganglioside cell association

(*a*) Time course of native GM1 ganglioside association to HeLa cells, at different glycolipid concentrations. ●, 1 μM; ◆, 5 μM; ■, 10 μM; ▲, 50 μM. Values are means ± S.D. Significance differences at 240 min: ▲ versus ■, ◆ or ●, $P < 0.01$; ■ versus ◆ or ●, $P < 0.05$; ● versus ◆, $P < 0.05$. (*b*) Time course of GM1 ganglioside molecular species association with HeLa cells, at glycolipid concentrations of 50 μM. ○, $C_{18:1}$-GM1; ◇, $C_{18:0}$-GM1; □, $C_{20:1}$-GM1; △, $C_{20:0}$-GM1. Significant differences at 240 min: ○ versus ◇, □ or △, $P < 0.05$; ◇ versus △, $P < 0.05$.

115 min for native GM1, 130 min for $C_{18:0}$-GM1, 160 min for $C_{20:0}$-GM1, and 180 min for $C_{20:1}$-GM1. At the above incubation times almost all (99%) of the cell-bound radioactivity was recovered in the aqueous phase of the lipid extract. Less than 5% of the radioactivity was found to be volatile and diffusible (possibly ^3H_2O). Radiochromatoscanning and autoradiography of the t.l.c. of the ganglioside extract showed the presence of a single radioactive peak having the same R_F as [^{3}H]GM1.

At incubation times longer than 8 h, the amount of volatile radioactivity increased considerably. Moreover, radiochromatoscanning of the t.l.c. of ganglioside extract showed the presence of other peaks besides [^{3}H]GM1.

Cyclic AMP content of HeLa cells

The average cyclic AMP content of HeLa cells grown in the presence of FCS and not treated with exogenous gangliosides was 2 pmol/mg of protein. This value was not modified by incubating the cells for the above-specified times either in the absence of FCS or in the presence of gangliosides. In order to set up the experimental conditions giving maximum activation of adenylate cyclase, cells were incubated with different amounts of forskolin, known to be a strong activator of adenylate cyclase (Seamon *et al.*, 1981). In a parallel experiment, cells were incubated with native GM1 in order to achieve an association of 1.3 nmol of ganglioside/mg of protein and then incubated in the presence of different amounts of CT. In both cases the amount of cyclic AMP accumulated by the cells was measured. A survey of the results (Fig. 2) shows that, after a 40 min incubation with 20 μg of CT/dish, the cyclic AMP content was 33 pmol/mg of

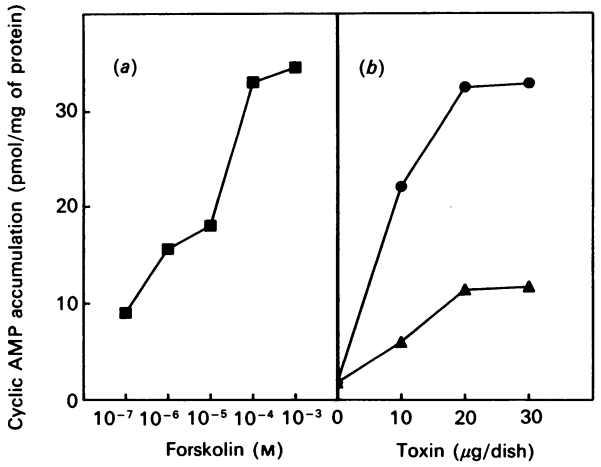

Fig. 2. Effects of GM1 ganglioside on cyclic AMP accumulation

(*a*) Cyclic AMP content of HeLa cells after 10 min of incubation with forskolin at different concentrations. (*b*) Cyclic AMP content of HeLa cells enriched (●) or not (▲) with native GM1 and incubated for 40 min in the presence of different amounts of CT.

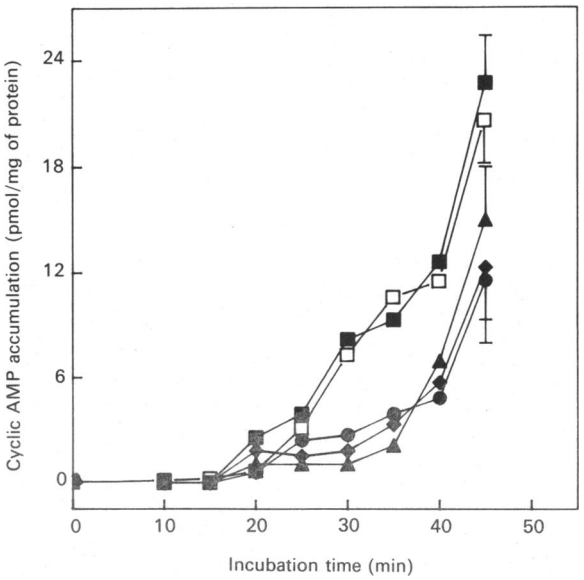

Fig. 3. Time course of cyclic AMP accumulation text on addition of CT to HeLa cells enriched with native GM1 or GM1 molecular species

■, $C_{18:1}$-GM1; ●, $C_{18:0}$-GM1; ▲, $C_{20:1}$-GM1; ◆, $C_{20:0}$-GM1; □, native GM1. Values are means ± S.D. Significant differences at 50 min: ■ versus ● or ◆, $P < 0.01$; □ versus ◆ or ●, $P < 0.05$.

protein, the same level as was reached using forskolin, thus indicating that maximal activation of adenylate cyclase has been realized. The time course of cyclic AMP accumulation was monitored after incubation of HeLa cells (control cells) in the presence of 20 μg of CT/dish. The same experiment was carried out on cells (sample cells) enriched with 1.3 nmol of native GM1 or GM1 molecular species. The difference between the cyclic AMP contents of sample and control cells was calculated at each incubation time (Fig. 3). The data show that the amount of cyclic AMP accumulated after a given time was higher in the cells enriched with $C_{18:1}$-GM1 than in the cells enriched with the other molecular species. The difference was as high as 33% after 40 min of incubation with the toxin. A remarkable difference is

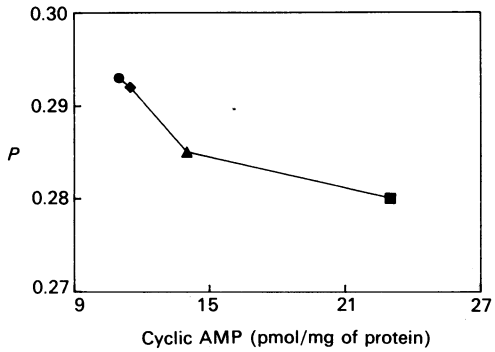

Fig. 4. Fluorescence anisotropy parameter (*P*) of HeLa cells enriched with GM1 molecular species versus cyclic AMP accumulation after 40 min of incubation with CT

■, $C_{18:1}$-GM1; ●, $C_{18:0}$-GM1; ▲, $C_{20:1}$-GM1; ◆, $C_{20:0}$-GM1.

evident in the duration of the lag time that precedes the accumulation of cyclic AMP, the shortest being recorded for the $C_{18:1}$ species. All ganglioside species exhibited similar rates of cyclic AMP accumulation after 35 min.

Accumulation of cyclic AMP in the presence of GM1 molecular species and forskolin

Cells were enriched with identical amounts of ganglioside molecular species, incubated in the presence of forskolin (0.1 mM), and the amount of cyclic AMP which had accumulated after 10 min was evaluated. No difference in cyclic AMP content was noted between cells enriched with any of the GM1 molecular species and those not enriched with GM1 (results not shown).

Fluorescence anisotropy experiments

Fluorescence anisotropy (*P*) of DPH was measured in order to evaluate the changes of membrane fluidity exerted by insertion of exogenous ganglioside molecular species. Fig. 4 plots the *P* values of cells enriched with GM1 molecular species versus the amount of cyclic AMP accumulated after 40 min of incubation with CT. As shown in Fig. 4, the lower the value of *P* recorded for the cells, the higher the amount of cyclic AMP that the cells accumulated.

DISCUSSION

The GM1 ganglioside content of HeLa cells is extremely low. We have detected an amount (20 pmol/mg of protein) that is between those reported by Fishman *et al.* (1980) and those reported by Markwell *et al.* (1984). This difference can be attributed to factors such as cell strain or culture conditions, which are known to influence the protein content of cells (Ryan *et al.*, 1972). Incubation of the cells in the presence of GM1 definitely leads to association of the glycolipid and renders the cells susceptible to CT (Fishman & Atikkan, 1979). The data herein reported show that GM1 molecular species having different LCB moieties have different time courses of association for the serum-stable form of association to cells. The shorter unsaturated LCB species ($C_{18:1}$-GM1) displayed faster association than the others. The difference can be attributed to a number of factors such as: (1) the different critical micellar concentrations of the different molecular species (Yohe *et al.*, 1976); (2) the fluidity of the membrane, modified to a certain extent by the insertion of the different molecular species (see Fig. 4); and (3) the interaction of gangliosides with cell membrane surface proteins (Sonnino *et al.*, 1989), which could modify the mode of association. Fishman *et al.* (1980) have previously reported that a synthetic derivative of GM1 carrying an acetyl group replacing the fatty acid showed a faster rate of association to cells than that of native GM1. The present work shows that the molecular species present in native ganglioside associate to cells in a fashion that is dependent on the LCB moiety. It is also worth noting that, after a given time of incubation, the proportion of the trypsin-stable form of associated ganglioside is smaller for the $C_{20:0}$ LCB species than for the others. In order to compare the influence of ceramide composition on the effects elicited by CT, the cells were enriched with identical amounts of the ganglioside molecular species which are present in native GM1. The experimental conditions chosen led to the incorporation of an amount of GM1 (1.3 nmol/mg of protein) which was greater by a 65-fold, than the amount of the endogenous GM1. It should be considered that the uptake of exogenous GM1 might occur not only to the plasma membrane but also to the cell interior, and, moreover, that the enrichment with exogenous GM1 may have indirect effects on the cells that could influence the results. However, no such observations have been reported previously in systems similar to ours, and it is generally agreed that the appearance of metabolic processing is indicative of ganglioside internalization (Fishman & Atikkan, 1979; Masserini *et al.*, 1990). Under our experimental conditions, almost all of the cell-associated radioactivity was recovered in the ganglioside extract and was still carried by GM1, suggesting that the major part of the associated ganglioside was still at the membrane level. For this reason we presumed that cellular responses elicited by interaction with CT could be attributed to the exogenously associated ganglioside and to differences in the different ceramide composition.

The data show that the ganglioside ceramide moiety of native GM1 does, in fact, affect the CT-triggered cyclic AMP accumulation of the cells. The overall mechanism could be affected by the ganglioside lipid portion at any stage. (1) The binding of the toxin to the cells could be influenced by the ganglioside ceramide composition, in analogy with other proteins (Masserini *et al.*, 1990), possibly reflecting different aggregation properties of the molecular species. The receptor density has been shown to affect the toxin action (Fishman & Atikkan, 1979). If this were the case, the results would reflect differences in the amounts of toxin bound or in the mode of toxin binding. (2) There may be differences in the membrane insertion of the catalytic A subunit and the splitting of the A1 peptide. It has been shown that insertion of proteins into the membrane is dependent on the bilayer lipid composition (Defrise-Quertain *et al.*, 1989). In this case the results would reflect a different mode of internalization or splitting of the hydrophobic subunit (A) of CT. (3) Differences may occur in the ADP-ribosylation of the regulatory G-protein or in the adenylate cyclase itself. It has been shown, for instance, that the cyclase activity is dependent on the bilayer fluidity (Stubbs & Smith, 1984). The fluorescence depolarization experiments herein reported show the existence of a direct relationship between membrane fluidity (which is affected by exogenous ganglioside insertion) and cyclic AMP content when cyclic nucleotide accumulation is triggered by CT, but not when accumulation is triggered by forskolin. These data suggest that the effect of membrane fluidity is not exerted directly upon adenylate cyclase but upon some of the stages described above, which are peculiar to the toxin. The observation that all ganglioside species exhibit similar rates of cyclic AMP accumulation after the lag period would suggest that most of the effects of the different molecular species of GM1 are actually exerted during the lag period.

This work was supported in part by C.N.R., Rome, Italy (grant no. 8600090.04.115.20055).

REFERENCES

Chigorno, V., Pitto, M., Cardace, G., Acquotti, D., Kirschner, G., Sonnino, S., Ghidoni, G. & Tettamanti, G. (1985) Glycoconjugate J. **2**, 279–291

Chigorno, V., Cardace, G., Pitto, M., Sonnino, S., Ghidoni, R. & Tettamanti, G. (1986) Anal. Biochem. **153**, 283–294

Defrise-Quertain, F., Cabiaux, V., Vandenbranden, M., Wattiez, R., Falmagne, P. & Russychaert, J.-M. (1989) Biochemistry **28**, 3406–3413

Fishman, P. H. (1982) J. Membr. Biol. **69**, 85–97

Fishman, P. H. & Atikkan E. E. (1979) J. Membr. Biol. **54**, 51–60

Fishman, P. H., Moss, J. & Osborne, J. C. (1978) Biochemistry **17**, 711–716

Fishman, P. H., Pacusza, T., Hom, B. & Moss, J. (1980) J. Biol. Chem. **255**, 7657–7664

Ghidoni, R., Tettamanti, G. & Zambotti, V. (1974) Ital. J. Biochem. **23**, 320–328

Ghidoni, R., Trinchera, M., Venerando, B., Fiorilli, A., Sonnino, S. & Tettamanti, G. (1986) Biochem. J. **237**, 147–155

Henneberry, R. C., Fishman, P. H. & Freese, E. (1975) Cell **5**, 1–9

Holmgren, J. (1981) Nature (London) **292**, 413–417

Markwell, M. A., Fredman, P. & Svennerholm, L. (1984) Biochim. Biophys. Acta **775**, 4–16

Masserini, M. & Freire, E. (1986) Biochemistry **25**, 1043–1049

Masserini, M., Giuliani, A., Palestini, P., Acquotti, D., Pitto, M., Chigorno, V. & Tettamanti, G. (1990) Biochemistry **29**, 697–701

Mekalanos, J. J., Collier, R. J. & Romig, W. R. (1979) J. Biol. Chem. **254**, 5855–5861

Moss, J., Fishman, P. H., Manganiello, V. C., Vaughan, M. & Brady, R. O. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 1034–1037

Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356

Received 9 February 1990/30 April 1990; accepted 21 May 1990

Phillips, H. J. (1973) in Tissue Culture Methods and Applications (Kruse, P. F. & Patterson, M. K., eds.), pp. 167–212, Academic Press, New York

Ryan, C. A., Lee, S. Y. & Nadler, H. L. (1972) Exp. Cell Res. **71**, 388–392

Schwarzmann, G., Hoffmann-Bleihauer, P., Shubert, J., Sandhoff, K. & Marsh, D. (1983) Biochemistry **22**, 5041–5048

Seamon, K. B., Padgett, W. & Daly, J. W. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 3363–3367

Shinitzky, M. & Barenholz, Y. (1978) Biochim. Biophys. Acta **515**, 367–394

Sonnino, S., Ghidoni, R., Galli, G. & Tettamanti, G. (1978) J. Neurochem. **31**, 947–956

Sonnino, S., Ghidoni, R., Gazzotti, G., Kirschner, G., Galli, G. & Tettamanti, G. (1984) J. Lipid Res. **258**, 620–629

Sonnino, S., Chigorno, V., Acquotti, D., Pitto, M., Kirschner, G. & Tettamanti, G. (1989) Biochemistry **28**, 77–81

Stubbs, C. D. & Smith, A. D. (1984) Biochim. Biophys. Acta **779**, 89–136

Suzuki, Y. & Suzuki, K. (1972) J. Lipid Res. **13**, 687–690

Svennerholm, L. (1957) Biochim. Biophys. Acta **13**, 856–858

Tettamanti, G., Bonali, F., Marchesini, S. & Zambotti, V. (1973) Biochim. Biophys. Acta **296**, 160–170

Tomasi, M., Battistini, A., Araco, A., Roda, L. G. & D'Agnolo, G. (1979) Eur. J. Biochem. **93**, 621–627

Trinchera, M., Wiesmann, U., Pitto, M., Acquotti, D. & Ghidoni, R. (1988) Biochem. J. **252**, 375–379

van Heyningen, S. (1983) in Membrane Receptors (Kleinzeller, A. & Richard, B., eds.), pp. 445–471, Academic Press, New York

Wiegandt, H., Ziegler, W., Staerk, J., Kraft, T., Ronneberger, H. J., Zilc, H., Karlsson, K.-A. & Samuelsson, B. E. (1976) Hoppe-Seyler's Z. Physiol. Chem. **357**, 1637–1646

Yohe, M. C., Roark, D. E. & Rosenberg, A. (1976) J. Biol. Chem. **251**, 7083–7087