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INHIBITORS OF PROTEIN SYNTHESIS BLOCK ACTION OF CHOLERA TOXIN

Jörg Hagmann and Peter H. Fishman

The Membrane Biochemistry Section, Developmental & Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

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SUMMARY: Prior treatment of macrophages with cycloheximide blocked the activation of adenylate cyclase by choleragen. The effect of cycloheximide was time and dose dependent and also caused by puromycin. Toxin receptors and the catalytic and regulatory components of the cyclase were still present. As degradation and generation of the A_1 subunit of choleragen was also blocked, we propose the existence of a membrane component that mediates the translocation of choleragen across the membrane.

Choleragen (CT)[1] activates adenylate cyclase after binding to specific cell surface receptors (reviewed in 1-3). In contrast to hormones, which exert their effect immediately after binding to their receptors, CT activates adenylate cyclase in intact cells after a characteristic lag period. The lag presumably reflects the fact that CT (or its active subunit) has to penetrate the membrane in order to reach its target site (1-4). Although the kinetics of the lag phase have been described in detail (4), relatively little is known about the mechanism of the translocation of the toxin. It has been proposed that a conformational change imparted to the toxin by multivalent binding of its B component to the receptor GM1 causes the release of the A component which can then penetrate the membrane (3). This hypothesis is partially supported by the observed changes in the fluorescence and circular dichroic spectra of CT and its B component upon binding to the oligosaccharide of GM1 (5).

Here we report that prior treatment of macrophages with inhibitors of protein synthesis prevents the activation of adenylate cyclase by CT. The block

[1]Abbreviations: CT, cholera toxin; cAMP, adenosine 3',5'-monophosphate; GM1, galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide; Iso, (-)-isoproterenol; PGE_1, prostaglandin E_1; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Gpp(NH)p, guanyl-5'-yl-imidodiphosphate.

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Table 1

EFFECT OF CYCLOHEXIMIDE AND PUROMYCIN ON ACCUMULATION OF cAMP IN MACROPHAGES

Additions	Control cells	Cycloheximide-treated cells	Puromycin-treated cells
	(pmol of cAMP per mg protein)		
None	116 ± 22	232 ± 4	272 ± 7
Iso (1 μM)	839 ± 91	1200 ± 100	1290 ± 369
PGE_1 (10 μM)	2480 ± 250	1660 ± 23	839 ± 76
CT (5 nM)	1030 ± 130	250 ± 11	266 ± 21

Cells were incubated with and without cycloheximide (1 μg/ml) or puromycin (4 μg/ml) for 12 h, stimulated with hormones for 10 min or with CT for 1 h and analyzed for intracellular cAMP content as described previously (7). Values represent the mean ± S.D. of triplicate determinations. Qualitatively identical results have been obtained in 5 similar experiments.

occurs between the binding and activation steps and implies the participation of a protein in the translocation of the A component of the toxin.

MATERIALS AND METHODS: Cycloheximide, puromycin and DNAase I were obtained from Sigma. CT was from Schwartz/Mann and was iodinated as described (6). Peritoneal macrophages from male Hartley guinea pigs (Charles River Labs) were induced with thioglycollate and cultured in serum-free medium as described (7). Indomethacin (3 μM) was always present to prevent prostaglandin production. cAMP accumulation, adenylate cyclase activity and ^{125}I-CT binding were determined as described (7). Protein synthesis was assayed by incubating cells in 35-mm wells with 2.7 μCi of L-[U-^{14}C]-Leu (290 Ci/mol from New England Nuclear) in 2 ml of medium for 14 h; ^{14}C precipitated by 5% trichloroacetic acid (TCA) then was determined (8). Degradation of ^{125}I-CT was measured by incubating cells in 35-mm wells with 0.2 nM ^{125}I-CT (5×10^5 cpm) in 1 ml of medium for 0.5 h at 22°C. The cells then were washed and incubated with fresh medium for the indicated times when TCA-soluble ^{125}I in the medium was assayed. Generation of A_1 was determined by incubating cells with 0.2 nM ^{125}I-CT (1.5×10^6 cpm/ml) at 4°C for 30 min. The cells then were washed and incubated in fresh medium for the indicated times at 37°C. The cells were washed, lysed in 10 mM Tris-HCl (pH 7.4), 0.25 mM $MgCl_2$, 2 mM phenylmethylsulfonylfluoride and 0.1% DNAase I for 5 min at 20°C, dissolved by adding SDS to 1% and analyzed on 12.5% SDS-polyacrylamide gels (9).

RESULTS: Macrophages exposed for 12 h to cycloheximide did not accumulate cAMP when stimulated with CT (Table 1). Basal and Iso-stimulated levels were enhanced somewhat while the response to PGE_1 was inhibited by 30-40%. Puromycin gave similar results. The response to CT was inhibited by cycloheximide in a time and dose-dependent manner (Fig. 1). The potentiation observed at 1 h was significant. A similar potentiation was observed in rat glioma C6 cells exposed

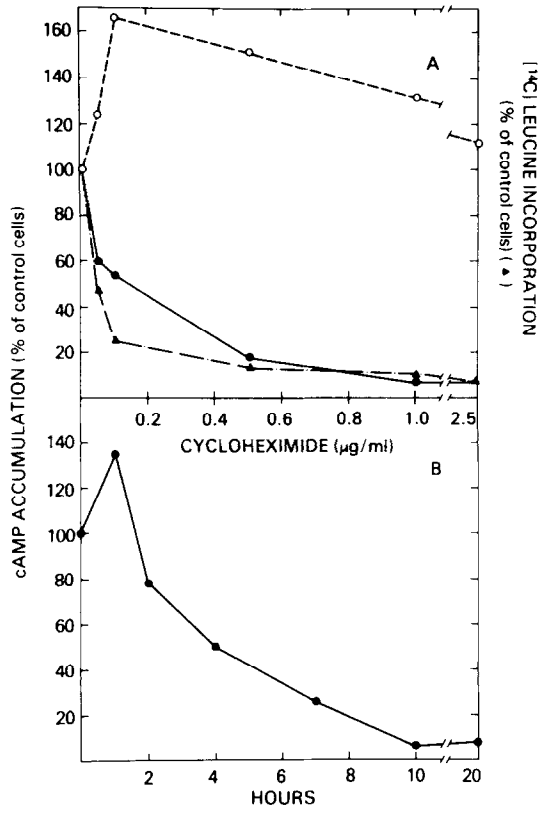

Fig. 1. Effect of cycloheximide on stimulation of cAMP accumulation and inhibition of protein synthesis in macrophages. A - Cells were incubated for 12 h with the indicated concentrations of cycloheximide and then analyzed for TCA-precipitable [^{14}C]-Leu incorporation or assayed for stimulation of cAMP accumulation by CT or Iso as indicated in Table 1. B - Cells were incubated with 1 μg /ml cycloheximide for the indicated times and then assayed for for CT-stimulated cAMP accumulation. CT (•); iso (○).

to cycloheximide for short times (10)[2]. When C6 cells or human fibroblasts were exposed to cycloheximide for 15 h, the response to CT was inhibited >50% (data not shown). Inhibition of CT action by increasing amounts of cycloheximide parallelled inhibition of protein synthesis (Fig. 1A).

Cycloheximide-treated macrophages bound more CT than did control cells (Table 2). By 5 h, binding had increased substantially whereas binding to control cells remained constant for up to 14 h (data not shown). When cyclohex-

[2]Although the mechanism involved in the potentiation is unknown, we observed no increase in specific toxin binding sites on either macrophages or rat glioma C6 cells at these early times.

Table 2

EFFECT OF CYCLOHEXIMIDE TREATMENT ON CT BINDING AND ADENYLATE CYCLASE ACTIVATION

Treatment	^{125}I-CT Specifically Bound	Adenylate Cyclase Activity	
		control cells	CT-treated cells
	(fmol/mg protein)	(pmol/mg protein x min)	
None	57.4	165	366
Cycloheximide	238	59	52

Macrophages were incubated for 12 h in the presence and absence of cycloheximide (1 μg/ml) and then assayed for specific ^{125}I-CT binding or treated with and without 5 nM CT for 1 h and assayed for adenylate cyclase activity. Cyclase assays included 10 μM GTP. Values are the mean of triplicate determinations which varied less than 10%.

imide-treated macrophages were incubated with CT, adenylate cyclase was not activated (Table 2). The enzyme from both control and treated cells, however, was stimulated by PGE_1, NaF, guanine nucleotides and activated CT (Table 3). Some activation by the latter agent was observed even in the absence of added NAD indicating that failure of CT to activate cyclase in intact cells was not due to NAD depletion. Although the absolute enzyme activites were lower in membranes

Table 3

ADENYLATE CYCLASE ACTIVITY OF CELLS TREATED WITH CYCLOHEXIMIDE

Additions	Adenylate Cyclase Activity (pmol cAMP/mg protein x min) Control cells	Cycloheximide-treated cells
None	109 (1.0)	34 (1.0)
GTP (10 μM)	165 (1.5)	59 (1.7)
GTP + PGE_1 (10 μM)	278 (2.5)	111 (3.3)
Gpp(NH)p (10 μM)	307 (2.8)	224 (6.6)
NaF (10 mM)	340 (3.1)	196 (5.8)
NAD*	179 (1.0)	79 (1.0)
NAD + CT*	263 (1.5)	178 (2.3)
None*	145 (1.0)	60 (1.0)
CT*	209 (1.4)	119 (2.0)

Cells were incubated for 12 h with or without 1 μg/ml of cycloheximide and assayed for adenylate cyclase activity (7). The results represent the average of two experiments. The values differed by less than 10%. Values in parentheses represent fold stimulation.

* CT was activated by incubating a solution containing 10 μM toxin, 10 mM dithiothreitol, 135 mM NaCl, 0.4 mg/ml ovalbumin and 40 mM Hepes (pH 7.5) for 10 min at 34°C. Membranes were incubated with 10 μM GTP and 1 μM activated CT and 1 mM NAD as indicated for 10 min at 34°C before assaying for enzyme activity.

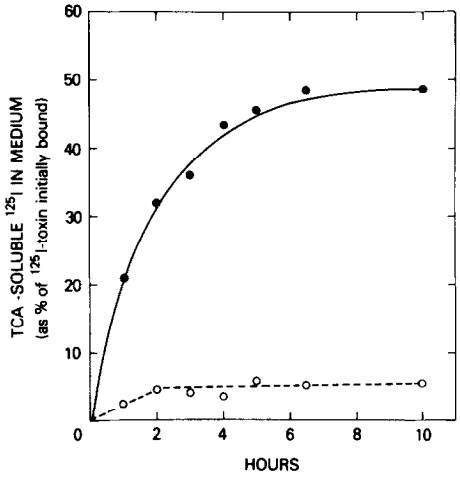

Fig. 2. Degradation of ^{125}I-CT by macrophages. Cells were incubated for 14 h in the presence (o) and absence (•) of 1 μg/ml cycloheximide and then assayed for the ability to degrade ^{125}I-CT as described under methods. Values are expressed as the % of ^{125}I-CT initially bound (26,601 and 49,032 cpm/well for control and for treated cells, respectively).

from cycloheximide-treated cells, the fold stimulation was similar for both the control and treated cells. As the drug blocks protein synthesis, the slow loss of adenylate cyclase components is not unexpected.

Degradation of ^{125}I-CT reached a plateau after 8 h when about 50% of the initially bound label appeared in the medium as TCA-soluble material (Fig. 2); cell-bound ^{125}I-CT declined concomitantly. Cells previously treated with cycloheximide, however, showed no accumulation of degraded toxin in the medium and the amount of cell-bound ^{125}I-CT remained at the initial level (Fig. 2).

The disulfide bridge linking the A_1 and A_2 subunits of the A component of CT has to be cleaved for A_1 to become active (11,12). We therefore attempted to determine whether the appearance of A_1 could be blocked by treating the cells with cycloheximide. Figure 3 shows the result of one of several experiments. After exposing intact cells to ^{125}I-CT for 30 min, label which comigrated with A_1 on the gel appeared in control cells but not in cycloheximide-treated cells. No increase in A_1 was observed after 5 min exposure in either cells (Fig. 3A). A_1 was detected in control cells by 15 min (Table 4) which coincides with the time course of CT action in macrophages (7). In contrast, the proportion of

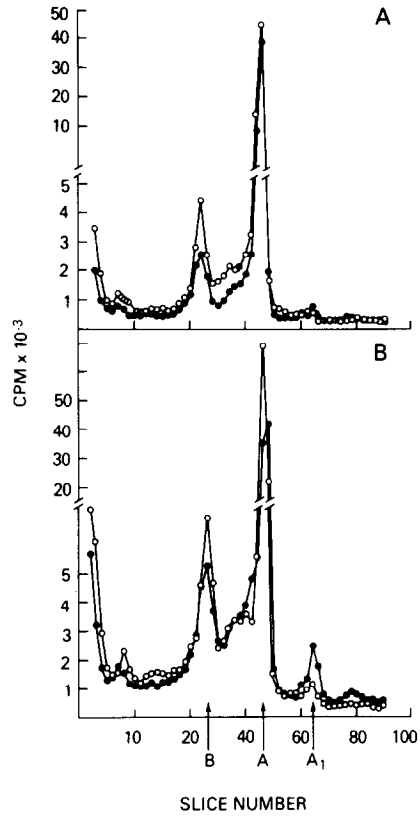

Fig. 3. Generation of A_1 in macrophages. Control cells (•) and cells treated for 12 h with 1 μg/ml of cycloheximide (○) were incubated with ^{125}I-CT at 4°C for 30 min. The cells then were washed, incubated at 37°C for 5 (A) or 30 (B) min, and analyzed for radioactivity by SDS-polyacrylamide gel electrophoresis as described under methods. Arrows indicate the location of the B (Mr = 56,000), the A (Mr = 28,000) and the A_1 (Mr = 21,000) components of CT.

cell-bound radioactivity that migrated as A_1 on the gels did not increase above the zero time control value in cycloheximide-treated cells even after 30 min. The small amount of A_1 (0.5% of the total CT bound) present in cycloheximide-treated cells appears to be due to non-specific reduction as it was present at zero time in both treated and control macrophages (Table 4). From the data in Figure 3 and Table 4, we estimate that a cell exposed to CT for 30 min at 37°C contains ∿100 molecules of A_1. This is consistent with kinetic studies of the activation of adenylate cyclase by CT which indicated that only a small fraction of the bound toxin was involved in the activation process (4,11).

Table 4

GENERATION OF A_1 SUBUNIT OF CT IN MACROPHAGES

Time at 37°C	Control cells		Cycloheximide-treated cells	
min	cpm	%*	cpm	%*
0**	370	0.58	396	0.43
5	362	0.30	379	0.26
15	1816	1.24	882	0.44
30	4240	1.84	1045	0.43

Control and cycloheximide-treated (1 µg/ml for 12 h) cells were incubated with ^{125}I-CT for 30 min at 4°C, washed and shifted to 37°C for the times indicated. Cells were then washed, homogenized, dissolved by adding SDS and analyzed by SDS-polyacrylamide gel electrophoresis as described under methods. The area of the gel corresponding to A_1 was counted (see Fig. 3).

* % of cpm applied to gel that corresponded to A_1.
** ^{125}I-CT was added to the cells after homogenization.

DISCUSSION: We have shown that blocking protein synthesis in macrophages for 12 h abolishes the effect of CT on adenylate cyclase. Inhibition was not due to depletion of the catalytic or the regulatory component of the enzyme since activated CT (A_1), hormones, guanine nucleotides and NaF stimulated the adenylate cyclase in membranes of cycloheximide-treated cells. Furthermore, the treated cells bound more ^{125}I-CT than did the control cells.

From the above results, we conclude that inhibition of protein synthesis blocks a step occurring between the binding of CT to its receptor and the ADP-ribosylation of the regulatory component of the cyclase. This conclusion is supported by two additional observations. First, degradation of ^{125}I-CT was blocked in cycloheximide-treated macrophages. Since degradation most likely occurs inside the cell, it follows that the toxin does not become exposed to degradative enzymes. Second, the disulfide bond between A_1 and A_2 must be reduced before ADP-ribosylation and activation of adenylate cyclase can occur (1,2,11, 12). This reduction step may be caused by intracellular reducing agents such as glutathione. Our experiments showed that in cycloheximide-treated cells the generation of A_1 was inhibited. We therefore propose that in these cells A

is not reduced because it does not penetrate the membrane. The inhibitory effect of cycloheximide on both the appearance of A_1 and the degradation of CT taken together indicate that in treated cells the toxin once bound to its receptor remains at the cell surface and is not translocated.

Our results could be explained by a model in which a membrane protein is involved in translocating CT (or its active subunit) in analogy to the "signal-hypothesis" put forward to explain the translocation of secretory proteins (13). In this regard, the A_2 subunit of CT may function as a "signal peptide". There is additional evidence to support our concept of a toxin translocator. Activation of adenylate cyclase by CT in thymoctes was inhibited by treating the cells first with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiamide (14). The cells still bound ^{125}I-CT and responded to Iso. Furthermore, dansylcadaverine but not other amines blocked the action and degradation of CT in neuroblastoma cells which still responded to PGE_1 (unpublished observations).

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