Vol. 120, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 30, 1984 Pages 700-706

INHIBITION OF ADP-RIBOSYLTRANSFERASE ACTIVITY OF CHOLERA TOXIN BY MDL 12330A AND CHLORPROMAZINE

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Received March 29, 1984

ADP-ribosylation by cholera toxin of the guanine nucleotide binding regulatory protein (G_s) of rat liver membrane adenylate cyclase was inhibited by 0.1-1 mM MDL 12330A or 0.1-1 mM chlorpromazine. Basal as well as cholera toxin activated adenylate cyclase activity in liver membranes was also inhibited by the two drugs. NAD glycohydrolase activity and self-ADP-ribosylation of cholera toxin were also inhibited by MDL 12330A and chlorpromazine. These effects of MDL 12330A and chlorpromazine may be related to their effects on cholera toxin-induced fluid secretion in vivo.

Cholera toxin activates adenylate cyclase by catalyzing the ADP-ribosylation of the guanine nucleotide-binding stimulatory subunit (G_s) of adenylate cyclase (1,2). Activation of adenylate cyclase in the intestinal mucosa by cholera toxin is responsible for the intense diarrhea and fluid loss of clinical cholera (3,4). A number of drugs have been described which ameliorate the effects of cholera toxin in various animal models (5-13), but the mechanism(s) of action of these drugs is largely obscure. One such agent, chlorpromazine, has as one of its effects the inhibition of adenylate cyclase (10) and has proved to be useful in treating intestinal hypersecretion in animal models (10,11) as well as human cholera (12). MDL 12330A (N-(cis-2-phenyl cyclopentyl)azacyclotridecan-2-imine hydrochloride) inhibited cholera toxin induced intestinal hypersecretion (13), gastric secretion (14) and adenylate cyclase activity in a number of tissues (15-18). The molecular mechanisms by which both MDL 12330A and chlorpromazine interfere with cholera toxin activation of adenylate cyclase have not been investigated. The present report shows that MDL 12330A and chlorpromazine inhibit the cholera toxin-dependent ADP-ribosylation of G_s in rat liver membranes. This effect of the compounds may be related to their efficacy in reducing the effects of cholera toxin on adenylate cyclase in vivo.

0006-291X/84 $1.50

MATERIALS AND METHODS

Preparation of Rat Liver Membranes. Liver membranes were prepared by a published procedure (19). Purified membranes were suspended in 1 mM $NaHCO_3$ at a concentration of 2-3 mg protein/ml and stored in liquid N_2. For each experiment liver membranes were thawed, centrifuged at 15,000 g for 10 min, washed twice with 10 mM Tris (pH 7.5) containing 0.1 mM EDTA and 1 mM dithiothreitol, and dispersed in the same buffer at a concentration of 2-3 mg protein/ml.

Activation of Cholera Toxin and Incubation with Membranes for Activation of Adenylate Cyclase. Cholera toxin (200 μg in 40 μl of 0.2 M NaCl with 50 mM Tris (pH 7.0), 1 mM EDTA and 3 mM NaN_3) was incubated in a total volume of 1 ml containing, in addition, 20 mM Hepes (pH 7.5), 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.2 mg/ml of ovalbumin for 10 min at 30°C as previously described (22). Membranes (1-1.5 mg of protein) in 1 ml of a solution containing 1 mM ATP, 1 mM NAD, 10 mM creatine phosphate, 100 units creatine kinase, and 2 mM GTP were added and incubation was continued for 10 min at 30°C. Membranes were then sedimented by centrifugation (15,000 g, 10 min), and washed three times with 10 mM Tris (pH 7.5) 0.1 mM EDTA and 1 mM dithiothreitol. For controls, another sample of membranes was treated identically, except that cholera toxin was omitted from the 10 min incubation at 30°C.

ADP-Ribosylation of Membranes. ADP-Ribosylation of membranes was carried out under conditions previously shown to be optimal for specific labelling of peptides linked to adenylate cyclase (21,22). Cholera toxin (500 μg) was activated by incubation with 20 mM dithiothreitol in 100 mM glycine buffer (pH 8.0) for 10 min at 30°C prior to addition to membranes. Membranes (500-600 μg protein) were incubated for 30 min at 30°C in 250 μl of 50 mM glycine (pH 8.0) containing 20 mM thymidine, 4 mM GTP, 20 μM [α-^{32}P]NAD and activated cholera toxin (250 μg/ml). ADP-ribosylation was terminated by addition of 1 ml of 10% trichloroacetic acid to precipitate membrane proteins. Proteins were sedimented by centrifugation (15,000 g, 10 min), the pellet was washed twice with 50 mM glycine (pH 8.0) and then dissolved in 2% sodium dodecyl sulfate for electrophoresis.

Polyacrylamide gel Electrophoresis and Autoradiography. Solubilized proteins were subjected to electrophoresis on 8% polyacrylamide slab gels following the method of Laemmli (23). Gels were silver-stained using a published procedure (24), dried and then exposed to Kodak X-Omat AR film for 18-24 hr at -80°C for autoradiographic visualization of cholera toxin substrates.

Adenylate Cyclase. Adenylate cyclase was assayed in triplicate in a total volume of 0.1 ml containing 10 mM $MgCl_2$, 0.5 mM ATP, 7 x 10^5 CPM of [α-^{32}P]ATP, 50 mM glycine (pH 8.0), 1 mM EDTA, 0.5 mM aminophylline, 3 mM phospho(enol)-pyruvate, 1 unit of pyruvate kinase, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol and 30-40 μg of rat liver membrane proteins. Assays were conducted at 37°C for 15 min then terminated by addition of 100 μl of a solution containing 40 mM ATP, 1.4 mM cAMP, 50 mM Tris (pH 7.5), 2% SDS and [^{3}H]cAMP (4000-5000 cpm) and cAMP was isolated for radioassay as described by Salomon, et al. (25).

NAD Glycohydrolase Activity of Cholera Toxin. NAD glycohydrolase (NADase) activity was determined as previously described (26). Cholera toxin (100 μg) was incubated at 37°C for 2 hr in a volume of 0.3 ml containing 300 mM potassium phosphate (pH 7.0), 20 mM dithiothreitol and 1 mM [carbonyl-^{14}C]NAD. Reactions were terminated by applying 0.1 ml aliquots of the reaction mixture onto duplicate columns (0.5 cm x 4 cm) of Dowex-1 which were previously equilibrated with 20 mM Tris (pH 7.5). [^{14}C]Nicotinamide was eluted with five 1 ml portions of 20 mM Tris-HCl (pH 7.5) and 10 ml Aquasol was added for radioassay.

Self ADP-ribosylation of Cholera Toxin. Self ADP-ribosylation by cholera toxin was determined in a manner similar to a published procedure (29). Cholera toxin (100 μg) was incubated with 1 mM [^{32}P]NAD (2 μCi) for 2 h at 37°C in the presence or absence of 0.3 mM MDL 12330A in a volume of 0.3 ml. The reactions were terminated by adding 3 ml of cold 10% trichloracetic acid and the precipitates were collected by filtration on 0.45 μM Millipore filters. Filters were washed 5 times with 3 ml aliquots of 5% trichloroacetic acid, once with 3 ml absolute ethanol, once with 3 ml toluene and then air-dried before liquid scintillation counting in 10 ml Aquasol.

Protein was measured by the method of Lowry et al. (27) using bovine serum albumin as the standard.

Chemicals. [Carbonyl-^{14}C]NAD (50 mCi/mmol) was purchased from Amersham; [α-^{32}P]ATP (30 Ci/mmole) and [G-^{3}H]cAMP from New England Nuclear; cholera toxin from Schwarz-Mann or List Biological Laboratories; chlorpromazine and NAD from Sigma; AG1-X2 (100-200 mesh) and all gel electrophoresis chemicals from Bio-Rad. MDL 12330A was synthesized in our laboratories (28).

RESULTS AND DISCUSSION

The ADP-ribosylation of subunits (M_r = 42 K and 45 K daltons) of the guanine nucleotide binding stimulatory protein (G_s) of rat liver membrane adenylate cyclase was inhibited by 0.1-1 mM MDL 12330A or 0.1-1 mM chlorpromazine (Fig. 1). Light densitometry was done on the autoradiograph to determine

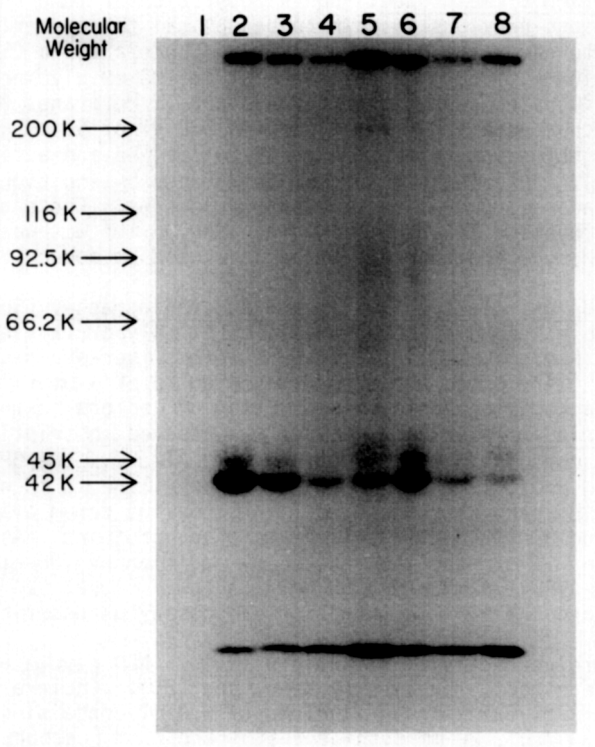

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ADP-ribosylated rat liver membrane proteins.

Cholera toxin-dependent ADP-ribosylation of membrane proteins was carried out as described in the Materials and Methods section in the presence or absence of various concentrations of MDL 12330A or chlorpromazine. Approximately 50 µg of protein from each membrane sample was loaded on the gels. Autoradiography on dried gels was carried out as described in Materials and Methods. Lanes 1, no additions; 2, cholera toxin (250 µg/ml); 3, cholera toxin plus 0.1 mM MDL 12330A; 4, cholera toxin plus 0.3 mM MDL 12330A; 5, cholera toxin plus 1 mM MDL 12330A; 6, cholera toxin plus 0.1 mM chlorpromazine; 7, cholera toxin plus 0.3 mM chlorpromazine; 8, cholera toxin plus 1 mM chlorpromazine. Protein markers and their molecular weights were myosin, 200,000; β-galactosidase, 116,000; phosphorylase B, 92,500; bovine serum albumin, 66,200 and ovalbumin, 45,000.

TABLE 1 Inhibition of Cholera Toxin-Dependent ADP-Ribosylation of Rat Liver Membrane M_r = 42,000 protein by MDL 12330A and Chlorpromazine

Cholera Toxin	MDL 12330A (mM)	Chlorpromazine (mM)	Relative Labelling Intensity
+			100
+	0.1		72
+	0.3		21
+	1.0		20
+		0.1	96
+		0.3	21
+		1.0	15

Rat liver membranes (500-600 μg protein) were incubated for 30 min at 30°C in the presence of activated cholera toxin (250 μg/ml) and [^{32}P]NAD as described in Materials and Methods. Trichloroacetic acid precipitated proteins were solubilized in sodium dodecyl sulfate and subjected to electrophoresis on 8% polyacrylamide slab gels after which the gels were dried and autoradiography was done. Labelling intensities of the M_r = 42,000 proteins were determined by light densitometry done on the autoradiograph shown in Figure 1.

the extent of inhibition in the ADP-ribosylated protein bands (Table 1). ADP-ribosylation of G_s in bovine brain and human erythrocyte membranes was inhibited approximately to the same extent as in rat liver membranes (data not shown).

Basal adenylate cyclase activity of rat liver membranes was inhibited by MDL 12330A and chlorpromazine when the drugs were added to the cyclase assay (Table 2). Cholera toxin-activated adenylate cyclase exhibited a biphasic response to MDL 12330A and chlorpromazine whether the drugs were added to the cyclase assay or to the initial incubation of membranes with cholera toxin (Table 2). Basal and cholera toxin-activated bovine brain adenylate cyclase demonstrated similar responses to MDL 12330A and chlorpromazine (data not shown). Biphasic responses of other membrane-bound enzymes to MDL 12330A have previously been reported (16). It was suggested by others that MDL 12330A inhibited adenylate cyclase through interaction with the catalytic site of the cyclase (15), but the biphasic nature of the drug effects reported here suggests that the response of cyclase to the drug is more complex.

As shown in Table 3, the NADase activity of cholera toxin was progressively inhibited by increasing concentrations of MDL 12330A or chlorpromazine. The NADase activity of diphtheria toxin was not inhibited by either MDL 12330A or chlorpromazine (data not shown) suggesting that inhibition of the reaction was

TABLE 2 Effects of MDL 12330 and Chlorpromazine on Basal and Choler Toxin-Stimulated Adenylate Cyclase in Rat Liver Membranes

Additions to Incubation I			Additions to Assay		Adenylate Cyclase	
CT	MDL 12330A (mM)	CPZ (mM)	MDL 12330A (mM)	CPZ (mM)	(pmol·15 min^{-1}·mg $protein^{-1}$)	
0					88	(100)
0			0.1		41	(47)
0			0.3		18	(20)
0			1.0		4	(5)
0				0.1	35	(40)
0				0.3	24	(27)
0				1.0	7	(8)
+					195	(100)
+			0.1		285	(146)
+			0.3		75	(38)
+			1.0		6	(3)
+				0.1	129	(66)
+				0.3	99	(51)
+				1.0	54	(28)
+	0.1				273	(140)
+	0.3				181	(93)
+	1.0				93	(48)
+		0.1			249	(128)
+		0.3			188	(96)
+		1.0			76	(39)

Rat liver membranes were isolated as described in Materials and Methods and incubated at 30°C for 10 min (Incubation I) with or without activated cholera toxin (CT) in the presence or absence of the indicated concentrations of MDL 12330A or chlorpromazine (CPZ). Membranes were then washed free of cholera toxin and drugs in preparation for the adenylate cyclase assay. Values for adenylate cylcase activity represent the means of triplicate assays. Numbers in parentheses represent percent of activity of membranes not exposed to drugs.

TABLE 3 Inhibition of NAD-glycohydrolase (NADase) Activity of Cholera Toxin by MDL 12330A or Chlorpromazine

Drug	Concentrations (mM)	NADase Activity (nmol·min^{-1}·mg cholera $toxin^{-1}$)
	0	1.43
MDL 12330A	0.1	0.89
	0.3	0.38
	1.0	0.057
Chlorpromazine	0.1	1.01
	0.3	0.47
	1.0	0.099

Cholera toxin (100 μg) was incubated for 100 min at 37°C with 1 mM [carbonyl-^{14}C]NAD (97,000 cpm/assay) and the indicated concentrations of either MDL 12330A or chlorpromazine. Reactions were terminated by pipetting duplicate 0.1 ml samples of the reaction mix onto Dowex 1 columns and product was eluted as described in Materials and Methods.

not due to interaction with the substrates of the NADase reaction nor was inhibition due merely to denaturation of the enzymes. Additionally, MDL 12330A was found to significantly inhibit the transfer of [^{32}P]ADP-ribose from NAD to cholera toxin catalyzed by cholera toxin itself; 1450 pmol [^{32}P]ADP-ribose/mg cholera toxin being incorporated in the absence of MDL 12330A and 540 pmol [^{32}P]ADP-ribose/mg cholera toxin being incorporated in the presence of 0.3 mM MDL 12330A. Self ADP-ribosylation of the A_1 subunit of cholera toxin has been shown to increase the enzymatic activity of that sub-unit (29).

While there are certainly other effects of both MDL 12330A (17,18) and chlorpromazine (11) that are relevant to the inhibition of cholera toxin effects in vivo, the inhibitory actions of MDL 12330A and chlorpromazine on ADP-ribosylation of G_s, cholera toxin-activated adenylate cyclase activity, cholera toxin NADase activity and cholera toxin self-ADP-ribosylation may contribute to the biological effects of the compounds in cholera toxin-induced hypersecretory states.

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