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ACTIVATION OF RAT LUNG PARTICULATE GUANYLATE CYCLASE BY CHOLESTEROL-SEQUESTERING AGENTS

Pushkaraj J. Lad*
John M. Dalton Research Center, University of Missouri-Columbia,
Columbia, Missouri 65211 U.S.A.

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SUMMARY: Naturally occurring cholesterol-sequestering agents, digitonin, cereolysin and streptolysin O, activated rat lung particulate guanylate cyclase. Particulate enzyme treated with digitonin and cereolysin was further activated by sodium nitroprusside. Digitonin and cereolysin lowered sodium nitroprusside activation of the rat lung soluable guanylate cyclase. Activation of the particulate guanylate cyclase by digitonin and cereolysin was not due to the solubilization of the enzyme.

INTRODUCTION

Certain membrane active toxins and natural products act by interacting with membrane lipid components. The lipid components of cell membrane (phospholipid and cholesterol) have been implicated in the modulation of many membrane-associated enzyme, including guanylate cyclase [GTP pyrophosphate lyase (cyclising) EC 4.6.1.2] which catalyses synthesis of 3',5'-cyclic GMP (cGMP) from GTP. Melittin, a cytotoxic polypeptide from bee venom and antibiotic polypeptides alamethicin and gramicidin S activate membrane-associated guanylate cyclase (1-2) by interacting with phospholipids. Filipin, a cholesterol sequestering polyene, also activates rat lung particulate guanylate cyclase (3). Here, stimulatory effects of particulate guanylate cyclase by naturally occurring agents, digitonin, cereolysin and streptolysin O are reported. Digitonin interacts with cholesterol to form a tight complex (4). Streptolysin O (from *Streptococcus pyogenes*) (5) and cereolysin (from *Bacillus cereus*) (6) are oxygen labile hemolytic peptides which are activated by thiol-treatment. These toxins have a common mode of action in altering membrane structure; cholesterol is the suggested membrane binding site for them (7).

*Present address: The Salk Institute, P. O. Box 85800, San Diego, CA 92138.

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MATERIALS AND METHODS

GTP was purchased from P. L. Biochemicals, and [α-^{32}P]GTP and [^{3}H]cyclic GMP were obtained from New England Nuclear. Cyclic GMP, creatine phosphate, creatine phosphokinase, digitonin and other unspecified biochemicals were from Sigma. Bio-Gel A-5m was obtained from Bio-Rad. Cereolysin and streptolysin O were generous gifts of Dr. Alan Bernheimer, New York University Medical Center & Dr. James Duncan of Northwestern University, respectively.

Enzyme Preparation. Male rats weighing in the range of 100-150 g were decapitated and lungs were perfused *in situ* with 10 ml of homogenizing buffer (50 mM Tris-HCl, 0.5 mM Na_2 EDTA, 10 mM 2-mercaptoethanol, pH 7.6) by injecting it in the right ventricle. The lungs were removed and washed twice, blotted, placed in 3 volumes of homogenizing buffer and homogenized for 10 seconds at 10,000 rev/min with a Willems Polytron equipped with a PT 20 ST generator. The homogenate was filtered through a 40-mesh stainless steel wire screen and centrifuged for 10 min at 8000 X g, after which the supernatant solution was centrifuged at 160,900 X g for 40 min. The supernatant was used as the soluble enzyme and the pellet (microsomes) was chromatographed on a Bio-Gel A-5m column (1.6 X 15-cm). The column was eluted with 50 mM Tris-HCl, pH 7.6 buffer containing 30 mM 2-mercaptoethanol. The void volume fractions were pooled and used as the particulate enzyme.

Assays and Biochemical Determinations. Guanylate cyclase assays were performed by determining the conversion of [α-^{32}P]GTP to [α-^{32}P]cyclic GMP. The assay mix contained 1.2 mM GTP, 6 mM $MnCl_2$, 5 mM cyclic GMP, 50 mM Tris-HCl, 15 mM creatine phosphate and 10 units of creatine phosphokinase (8). Enzyme activity was assayed for 5 to 6 min at 37°C with 10-30 µg protein. The [α-^{32}P]cyclic GMP formed was separated from substrate by sequential chromatography on Dowex 50 and neutral aluminum oxide columns according to White and Karr (9). Proteins were determined by the method of Lowry *et al.* (10) after precipitation with silicotungstic acid (11). Bovine serum albumin was used as the standard. Specific activities are expressed as picomoles cyclic GMP formed/min/mg protein. All assays were in triplicate, and the activities are given as mean ± standard error of the mean. Data presented here are representative of at least 2 or 3 experiments.

RESULTS AND DISCUSSION

Guanylate cyclase is found in particulate and soluble fractions of rat lung homogenate (12,13). Most of the particulate enzyme is latent; non-ionic detergents (Lubrol PX or Triton X-100) and lysolecithin increase the activity 8-12 fold (14). The soluble enzyme from rat lung and other sources is activated by nitric oxide or nitric oxide generating compounds like nitroprusside and nitrosoguanidine derivatives (15,16). However, these compounds cause only small stimulation of the particulate enzymes (15). Digitonin treatment changed the rat lung particulate guanylate cyclase activity (Fig. 1). Digitonin activated the enzyme at low concentrations, the optimal activation being evident in a narrow concentration range (0.02-0.04%), which is far below the concentration usually used for solubilization of membrane

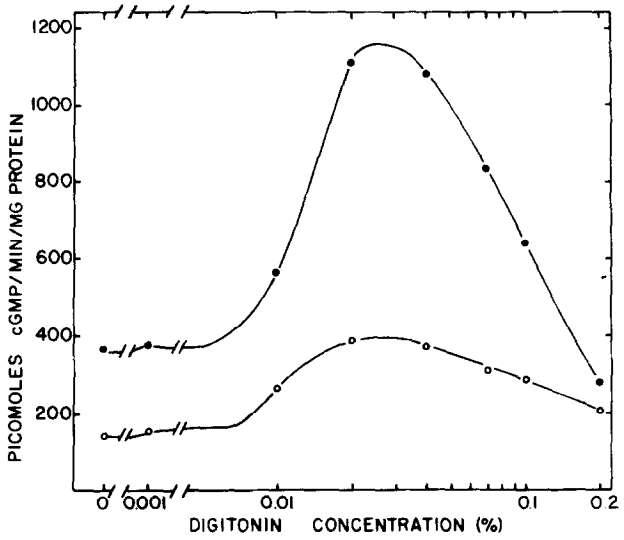

Fig. 1. The effect of digitonin on particulate guanylate cyclase activity. Enzyme was added to tubes containing different concentrations of digitonin and preincubated for 3 min at 37°C, then guanylate cyclase activity was assayed for 6 min at 37°C with (●) and without (○) 1 mM sodium nitroprusside.

proteins. The optimal activation was about 2.4 fold. Sodium nitroprusside (1 mM) activated the basal and digitonin-treated membrane-associated enzyme 2.4 fold and 3.0 fold, respectively. In some experiments, nitroprusside activated the digitonin-treated enzyme more than 3 fold. Digitonin and nitroprusside together caused 7-8 fold activation of the basal activity. Effects of bacterial toxins, cereolysin and streptolysin O on the particulate guanylate cyclase are shown in Table I. Cereolysin activated the enzyme about 5-6 fold, and this activity was further stimulated by nitroprusside. Cereolysin and nitroprusside together activated the enzyme about 11.8 fold. Streptolysin O produced some activation although to a lesser extent than cereolysin (Table I).

Effects of these agents cannot be explained by their lytic property. First, lytic δ toxin (from *Staphylococcus aureus*) did not alter guanylate cyclase activity (data not shown). Second, neither digitonin nor cerelolysin solubilized the guanylate cyclase activity. Third, when a digitonin-treated particulate preparation was chromatographed on a Bio-Gel A-5m column, most of

Table I. Effect of cereolysin and streptolysin O on particulate guanylate cyclase

Agent	Concentration HU	picomoles cGMP formed/min/mg - SNP	picomoles cGMP formed/min/mg + SNP
None	-	117.0 ± 10.0	300.2 ± 7.0
Cereolysin	100	663.3 ± 8.0	1506.0 ± 18.4
None	-	143.4 ± 2.1	363.3 ± 5.6
Streptolysin O	10	143.5 ± 5.7	389.8 ± 2.9
	100	216.9 ± 6.9	514.2 ± 2.1

Rat lung particulate fraction was treated with streptolysin O or cereolysin and assayed with and without 1 mM sodium nitroprusside (SNP) for 6 minutes at 37°C. The activity is expressed as mean ± standard error of mean. HU = hemolytic unit.

the enzymatic activity was eluted in the void volume as an insoluble enzyme. Furthermore, such enzyme was further activated by nitroprusside. Enzyme treated with detergents is not sensitive to nitroprusside. Nitroprusside stimulated particulate enzyme activity in the presence of non-ionic detergent Lubrol PX, at concentrations below 0.01%; above 0.01%, Lubrol PX produced a progressive increase in activity, which was not further stimulated by nitroprusside (Fig. 2). Lubrol PX like other non-ionic detergents increases the activity by solubilizing the particulate guanylate cyclase into non-sedimentable form (14,17).

These studies demonstrate that the natural agents which interact with cholesterol can modulate membrane bound guanylate cyclase. Most mammalian tissues contain some membrane-associated guanylate cyclase; in small intestine and stomach it is mostly in a membrane bound form. Cholesterol sequestering antibiotic, filipin seem to activate the membrane associated guanylate cyclase by increasing the membrane fluidity or increasing the membrane disorganization (18). Such a mechanism also may explain the activation of the particulate guanylate cyclase by the natural agents studied here. It is proposed that thiol-activated toxins bind to cholesterol in the membrane, the toxin-cholesterol complexes aggregate in the plane of

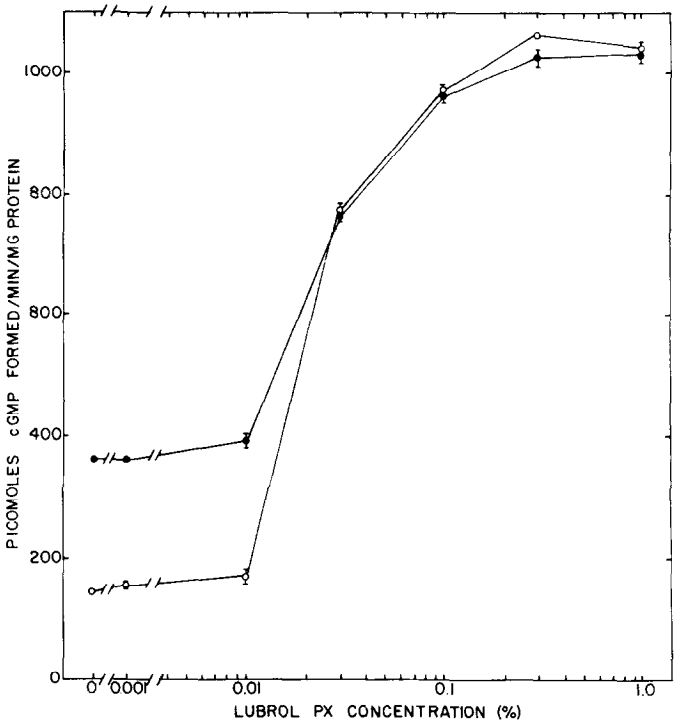

Fig. 2. The effect of Lubrol PX on sodium nitroprusside activation of particulate guanylate cyclase. The particulate enzyme was treated with different concentrations of Lubrol PX and assayed for activity at 37°C with (●) and without (○) 1 mM sodium nitroprusside.

the membrane and cause lysis. Lysis occurs because of increased membrane disorganization due to removal of cholesterol from its normal interaction with phospholipids (7,19). Cereolysin aggregates mostly in the hydrophobic core of the membrane (6), this may explain it being better activator of the guanylate cyclase which is a hydrophobic protein. Unlike filipin, streptolysin O and cereolysin do not cause the release of substances trapped in the liposome, the structural alterations produced by them seen only in the outer half of the hydrophobic layer of the membrane and do not transverse the membrane bilayer (6). If that is true for their action with rat lung membranes, then the results reported here argue against an unlikely possibility that increase in the guanylate cyclase activity is merely the soluble enzyme trapped inside the membrane vesicle.

Table II. Effect of digitonin and cereolysin on soluble guanylate cyclase

Agent	Concentration	picomoles cGMP formed/min/mg	
		- SNP	+ SNP
None	-	537.9 ± 19.9	8361.3 ± 791
Digitonin	0.003%	523.3 ± 23.1	7610.4 ± 165
	0.01 %	462.9 ± 4.9	7168.1 ± 174
	0.03 %	440.1 ± 14.3	4886.1 ± 626
	0.1 %	482.2 ± 11.1	2025.9 ± 244
Cereolysin	100 HU	464.5 ± 11.1	4127.9 ± 261

About 48 μg of soluble enzyme was treated with water, digitonin or cereolysin. Guanylate cyclase activity was determined at 37°C with and without 1 mM sodium nitroprusside (SNP). The activity is given as mean ± standard error of mean. HU = hemolytic unit.

Ability of these natural agents to modulate guanylate cyclase and probably other similar enzymes may partially explain their toxic nature. A specific functional role for cyclic GMP is unknown, but it has been suggested to play a role in smooth muscle relaxation, neuronal excitation and secretion of both small and large molecules. Therefore, the agents which modulate guanylate cyclase can impair some vital functions. Other bacterial toxins suggested to bind cholesterol, are tetanolysin, pneumolysin and thuringiolysin (7,19-21). A lytic peptide metridiolysin from sea anemone also is suggested to bind cholesterol (22-23).

Digitonin and cereolysin did not alter the basal soluble guanylate cyclase activity, but when treated with these agents it was less activated by nitroprusside (Table II). The decrease in nitroprusside activation due to digitonin was proportional to digitonin concentration. Enzyme treated with 0.1% digitonin was activated only 4.2 fold by nitroprusside as compared to 15.5 fold in the case of untreated enzyme. This may explain the biphasic effect of digitonin on the particulate enzyme seen in Fig. 1: (a) at low concentrations, cholesterol binding compounds increase the activity by changing fluidity; and (b) at higher concentrations, they decrease nitro-

prusside activation probably by interacting with the enzyme which has hydrophobic properties (14).

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