Biochimica et Biophysica Acta, 481 (1977) 734—740

BBA 68117

ACTIVATION OF LIVER GUANYLATE CYCLASE BY BILE SALTS AND CONTAMINANTS IN CRUDE SECRETIN AND PANCREOZYMIN PREPARATIONS

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(Received November 1st, 1976)

Summary

Crude preparations of secretin or pancreozymin increased and at higher concentrations decreased guanylate cyclase (GTP pyophosphate-lyase, EC 4.6.1.2) activity from soluble and particulate fractions of rat liver homogenates. Partially purified and synthetic secretin were without effect as was the biologically active octapeptide fragment of pancreozymin. The active contaminants in these preparations survived boiling, saponification, and treatment with phospholipase A, trypsin and neuraminidase C. The activity was extractable with chloroform/methanol and did not survive ashing. Eight bile salt contaminants in crude secretin were obtained with thin-layer chromatography. Two of the contaminating bile salts that increased liver particulate guanylate cyclase activity were identified as taurodeoxycholate and either glycochenodeoxycholate or glycodeoxycholate; taurocholate was inhibitory. The sodium salts of cholate, deoxycholate, chenodeoxycholate and their glycine- or taurine-conjugated forms either increased or decreased particulate and soluble rat liver guanylate cyclase activity depending upon their concentration. Thus, the previously reported stimulatory and inhibitory effects of secretin and pancreozymin preparations on guanylate cyclase activity are probably attributable to their bile salt contaminants.

Introduction

Since the early descriptions of guanylate cyclase (GTP pyrophosphate-lyase, EC 4.6.1.2) [1—3], some of the properties of this enzyme system have been reported. Most mammalian tissues have two or more forms of the enzyme with

Abbreviations: cyclic GMP, guanosine 3′,5′-monophosphate; cyclic AMP, adenosine 3′,5′-monophosphate; EDTA, ethylenediamine tetraacetic acid.

different kinetic and physical properties [4—7]. In comparison to adenylate cyclase relatively little is known about the mechanism(s) to regulate the activity of guanylate cyclase in tissues. Studies with regenerating liver, fetal liver, and tumors indicate that the activities of the soluble and particulate forms of the enzyme are regulated independently of one another in tissues [8,9]. A variety of hormones can increase cyclic GMP levels in different tissues [10—19]. Because of the many similarities of the cyclic AMP and cyclic GMP systems, hormonal regulation of guanylate cyclase has been expected. However, there have been very frew reports describing hormonal activation of the enzyme in cell-free systems. In most instances these observations have not been confirmed. Secretin [20] and pancreozymin (cholecystokinin) [18,19] have been reported to increase guanylate cyclase activity in some broken cell preparations. In this report the effects of crude preparations of these hormones are ascribed to bile salt contaminants. Partially purified and synthetic secretin did not activate guanylate cyclase. Similarly the biologically active octapeptide fragment of pancreozymin was inactive. Some bile salts which are present in crude preparations of these hormones can either increase or decrease the activity of guanylate cyclase in supernatant and particulate fractions of rat liver homogenates. Some of these observations have been reported in abstract form [21].

Materials and Methods

Male Sprague-Dawley rats weighing 200—250 g were decapitated and livers were removed quickly and rinsed with cold 0.25 M sucrose containing 5 mM Tris · HCl buffer (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol. Tissues were homogenized at 4°C in 11 volumes of the above buffer with a glass homogenizer fitted with a teflon pestle. Particulate and supernatant fractions of homogenates were separated by centrifugation at 105 000 × *g* for 60 min. Pellets were resuspended in a volume of buffer equal to that of original homogenates.

Guanylate cyclase activity was determined in reaction mixtures containing 50 mM Tris · HCl buffer, pH 7.6, 10 mM theophylline, 0.1 mM EDTA, 0.1 mM dithiothreitol, 7.5 mM creatine phosphate, and 14 μg of creatine phosphokinase (163 units/mg). Reactions were started by the addition of 4 mM $MnCl_2$ and 1 mM GTP in a final volume of 100 μl, incubated for 10 min at 37°C, terminated by the addition of 1 ml of 50 mM sodium acetate buffer (pH 4.0), and heated at 90°C for 3 min. Cyclic GMP formed was determined by the radioimmunoassay method of Steiner et al. [22] with some modification [14]. Values reported are means of duplicate or triplicate incubations from representative experiments.

Thin-layer chromatography was carried out with 0.25 mm silica gel F-254 plates (E. Merck, Darmstadt, Germany). Plates were prerun with a mixture of 1,2-dichloroethane/acetic acid/water (10 : 10 : 1, v/v), air dried, and heated at 120°C for 60 min. 30 units of crude secretin was applied as a 5 cm streak and plates were developed with the above system [23]. Areas of plates were extracted with chloroform/methanol (2 : 1, v/v). Solvent was removed at 40°C under reduced pressure and samples were suspended in 100 μl of water. 30 μl of these extracts were added to guanylate cyclase incubations. 3α-Hydroxy-

steroid was determined by the method of Turnberg and Anthony-Mote [24]. In brief, reaction mixtures contained 50 mM pyrophosphate buffer (pH 10.8), 2 mM NAD, and 0.1 unit of 3α-hydroxysteroid dehydrogenase (ICN Pharmaceutical Inc., Lot 0194). Reactions were carried out for 40 min at room temperature.

Crude secretin and pancreozymin were obtained from Sigma Co. (Lot 54C-0261 and Lot 34C-0050). Synthetic secretin was obtained from Schwartz Mann. Partially purified secretin was kindly provided by Eizai Pharmaceutical Co., Tokyo, Japan. The biologically active octapeptide fragment of pancreozymin was supplied by E.R. Squibb and Sons, Inc. Reagent grade solvents were obtained from Fisher Scientific Co. Glycodeoxycholate was purchased from Calbiochem. Other reagents were obtained from Sigma Co. or as described previously [4,8,14].

Results

At concentrations of 3—15 units/ml crude preparations of secretin markedly increased liver particulate guanylate cyclase activity (Fig. 1). Higher concentrations were inhibitory. Smaller stimulatory effects were observed with soluble guanylate cyclase. However, concentrations greater than 5—10 units/ml were also inhibitory with this preparation. Partially purified and synthetic secretin were without effects on particulate or soluble liver guanylate cyclase (Table I). Crude preparations of pancreozymin also increased liver soluble (5—10 units/ml) and particulate (10—30 units/ml) guanylate cyclase activities about 70% and 5-fold, respectively, as summarized in Table I. Higher concentrations were inhibitory. The biologically active octapeptide fragment of pancreozymin was without effect (Table I).

The stimulatory effects (at low concentrations) and inhibitory effects (at high concentrations) of crude secretin on particulate and soluble liver guanylate cyclase were not altered after heating preparations at 100°C for 30 min (not shown). Activity was also unaltered by saponification of crude secretin in 1 M NaOH at 100°C for 2 h. However, ashing at 500°C overnight resulted in loss of

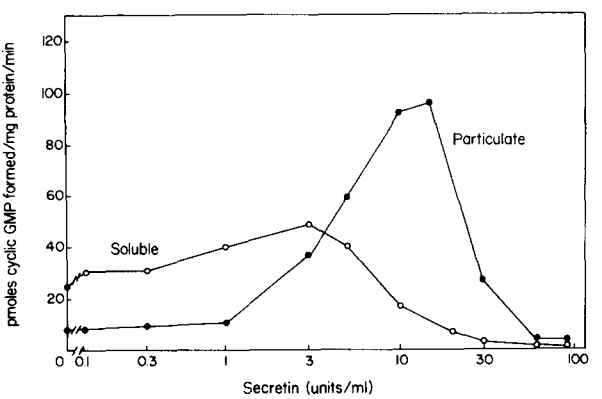

Fig. 1. Effect of crude secretin on rat liver guanylate cyclase. 105 000 × *g* particulate (●) and soluble (○) fractions of a rat liver homogenate were incubated as described with the concentrations of crude secretin indicated.

TABLE I

EFFECTS OF SECRETIN AND PANCREOZYMIN ON RAT LIVER GUANYLATE CYCLASE

Soluble and particulate guanylate cyclase activities in a rat liver homogenate were determined as described with the concentration of partially purified secretin, synthetic secretin, crude pancreozymin or its biologically active octapeptide at the concentrations indicated.

Preparation		Cyclic GMP formed (pmol/mg protein per min)	
		Soluble	Particulate
Expt. A			
Partially purified secretin (μg/ml)	0	49.5	11.9
	1	49.5	12.5
	10	51.4	11.7
	100	48.8	12.3
Expt. B			
Synthetic secretin (μg/ml)	0	25.9	5.1
	6	25.9	5.2
	20	27.6	5.2
	50	24.6	5.8
Expt. C			
Crude pancreozymin (units/ml)	0	36.5	7.2
	1	41.1	6.8
	5	58.0	7.2
	10	62.4	10.6
	30	2.4	36.4
	90	0.4	3.0
Expt. D			
Octapeptide (μg/ml)	0	25.5	5.1
	10	25.5	4.8
	30	29.0	4.8
	90	25.8	4.3

activity. These results indicated that the stimulatory and inhibitory effects of crude secretin preparations were not due to secretin or some contaminating metal ion. The stimulatory and inhibitory effects of crude secretin preparations were also unaltered with trypsin, phospholipase A, and neuraminidase C treatment (Table II).

Most of the activity on guanylate cyclase in crude secretin preparations was extracted with chloroform/methanol (2 : 1, v/v) and was soluble in both water and acetone (not shown). Thus, it appears that the active materials in secretin preparations had some properties of a lipid. Bile salts are added to intestinal extracts in order to precipitate secretin preparations [25]. The concentration of 3α-hydroxysteroids in crude secretin preparations that we tested was 285 nmol/unit of secretin. Crude pancreozymin preparations contained 165 nmol/unit of pancreozymin. Thin-layer chromatography of crude secretin preparations revealed eight bands with iodine vapor staining. Two of these areas (R_F 0.16 and 0.50) markedly increased rat liver particulate guanylate cyclase activity (Table III). These areas corresponded to taurodeoxycholate and glycodeoxycholate or glycochenodeoxycholate, respectively. Areas corresponding to taurocholate and an unknown material at the origin were inhibitory.

The effects of several bile salts on particulate and soluble rat liver guanylate

TABLE II

EFFECT OF ENZYMATIC DIGESTION OF CRUDE SECRETIN

After crude secretin was treated with the enzymes indicated at 37°C for 20 min, samples were heated at 95°C for 30 min before they were added to rat liver guanylate cyclase incubations. Values in parentheses indicate the percent of guanylate cyclase activity assayed without secretin.

Preparation	Crude secretin (units/ml)	Cyclic GMP formed (pmol/mg protein per min)				
		Experiment A		Experiment B		
		None	Phospholipase A (50 units/ml)	None	Trypsin (50 μg/ml)	Neuraminidase C (50 μg/ml)
Particulate guanylate cyclase	0	9.6(100)	11.1(100)	9.5(100)	10.0(100)	9.4(100)
	15	107.6(1120)	140.8(1268)	63.7(671)	60.2(602)	75.4(802)
	90	1.9(20)	1.8(16)	2.0(21)	2.2(22)	1.6(17)
Soluble guanylate cyclase	0	52.6(100)	60.5(100)	50.4(100)	49.2(100)	50.6(100)
	3	87.0(165)	92.4(153)	78.8(156)	60.6(123)	88.8(175)
	90	1.8(3)	1.7(3)	3.2(6.3)	2.6(5.3)	4.0(8)

cyclase activity are summarized in Table IV. 1—5 mM sodium deoxycholate and sodium chenodeoxycholate increased particulate guanylate cyclase activity and inhibited at higher concentrations (30 mM). Sodium cholate was less stimulatory. At 0.1—3 mM concentrations these agents were generally inhibitory with the soluble enzyme (Table IV). Sodium cholate was also less effective. The stimulatory and inhibitory effects of the three bile salts tested were similar when the glycine- or taurine-conjugated forms were used (not shown). Lecithin, another component of bile, had no stimulatory effects and was slightly inhibitory at 0.03—3 mM (not shown). None of these agents at the concentrations tested and carried into the radioimmunoassay had effects in the cyclic GMP radioimmunoassay.

TABLE III

THIN-LAYER CHROMATOGRAPHY PATTERN OF SECRETIN CONTAMINANTS THAT ALTER RAT LIVER GUANYLATE CYCLASE ACTIVITY

Crude secretin and known bile salts were chromatographed as described in Materials and Methods. Rat liver particulate guanylate cyclase activity is reported as percent of control activity assayed without thin-layer chromatography extracts which was 5.75 pmol/mg protein per min.

Bile salt marker	R_F	Guanylate cyclase (percent of control)
	0	16.2
Taurocholate	0.09	4.2
Taurodeoxycholate	0.16	1064.7
Taurochenodeoxycholate	0.24	129.7
Glycocholate	0.32	98.6
No marker	0.38	93.4
Glycodeoxycholate and glycochenodeoxycholate	0.50	682.1
Cholate	0.57	98.1

TABLE IV

EFFECT OF BILE SALTS ON RAT LIVER GUANYLATE CYCLASE ACTIVITY

Particulate and soluble rat liver guanylate cyclase were assayed as described in Materials and Methods with the concentrations of bile salts indicated. Values are reported as percent of control activity assayed without bile salts. Control activity was 31.7 and 9.1 pmol/mg protein per min for soluble and particulate preparations, respectively.

Bile salt	mM	Guanylate cyclase (percent of control)	
		Soluble	Particulate
Sodium cholate	0.1	90.1	—
	0.5	89.3	—
	1	—	87.0
	3	76.0	—
	5	—	95.7
	30	—	153.2
Sodium deoxycholate	0.1	83.4	—
	0.5	58.1	—
	1	—	154.3
	3	1.3	—
	5	—	299.4
	30	—	34.7
Sodium chenodeoxycholate	0.1	68.5	—
	0.5	128.1	—
	1	—	284.0
	3	1.8	—
	5	—	561.7
	30	—	34.8

Discussion

Crude preparations of secretin increased rat liver particulate guanylate cyclase activity 8—12-fold and soluble enzyme activity 30—70%. Crude pancreozymin also increased particulate and soluble rat liver guanylate cyclase activity. Synthetic or partially purified secretin and the biologically active octapeptide fragment of pancreozymin were without effects. The active contaminants in crude secretin preparations survived boiling, saponification in NaOH, and digestion with phospholipase A, trypsin and neuraminidase C. They were extractable with chloroform/methanol and did not survive ashing. Our crude secretin contained bile salts (285 nmol 3α-hydroxysteroid/unit secretin) that are added for preparation of secretin from intestinal extracts. A variety of bile salts were found in crude secretin preparations. Taurodeoxycholate and either glycochenodeoxycholate or glycodeoxycholate were identified as contaminants that increased rat liver particulate guanylate cyclase activity in a dose-dependent manner. Sodium cholate and its glycine or taurine conjugate were less effective.

The effects of bile salts are probably attributable to their properties as detergents. Cholic acid was the least effective bile salt tested and has a greater critical micellar concentration [26]. Thus, the stimulatory effects of bile salts may be analogous to the previously reported effects of Triton X-100 on guanylate cyclase activity [4,27,28]. The inhibitory effects at higher concentrations may be due to denaturation of guanylate cyclase, but this remains

to be established with an examination of the reversibility of the effect. Soluble rat liver guanylate cyclase was more sensitive to the inhibition. This may reflect the different properties of the soluble and particulate forms of the enzyme in liver and other tissues [4—7]. Thompson et al. [20] reported that partially purified secretin increased guanylate cyclase activity in an 18 000 × *g* supernatant fraction from rat liver in the range of 5—20 μg/ml; higher concentrations were inhibitory. While their preparation of secretin was thought to be quite pure, it seems likely that these effects can also be attributed to bile salt contaminants.

Acknowledgements

This work was supported by grants from the National Institutes of Health (AM 15316, HL 18260, AM 17042). F.M. is a recipient of a U.S.P.H.S. Research Career Development Award, AM 70456. The authors thank Joanne Holmes, Bing Chang and Robert Aldred for their technical assistance.

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