PURIFICATION OF GUANYLATE CYCLASE FROM HUMAN PLATELETS AND EFFECT OF ARACHIDONIC ACID PEROXIDE

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SUMMARY

Guanylate cyclase of human platelets was separated from cyclic nucleotide and GTP hydrolytic activities with a 104-fold purification over the homogenate. The purified guanylate cyclase preparation requires neither the GTP regenerating system nor cyclic GMP but is stimulated by about 2-fold by 2.5 mM cyclic GMP. The molecular weight of the enzyme was estimated as 180,000 and the Km value for GTP was 95 μM. Arachidonic acid peroxide stimulated the purified enzyme by increasing maximum velocity without changing Km value.

Guanylate cyclase is reportedly stimulated by nonionic detergents (1, 2), sodium azide (3), lysolecithin, phospholipase A_2 (4-6), sodium nitroprusside, nitroglycerin and nitric oxide (7). Recently, it was demonstrated that fatty acids stimulate guanylate cyclase activities from Balb 3T3 fibroblastes (8), rat fat cells (9) and human platelets (10, 11). In a previous study (12), we found that guanylate cyclase activity was stimulated by unsaturated fatty acid peroxides rather than fatty acids themselves and that the stimulation was apparently due to a peroxide-induced oxidation of -SH groups of the enzyme protein. Effects of various substances on guanylate cyclase have been studied and in most cases, a crude or a partially purified preparation which contained cyclic nucleotide and GTP hydrolytic activities was used. Therefore, these effects on guanylate cyclase may have been due to an indirect influence through cyclic nucleotide or GTP hydrolytic activity. To determine the direct effect of various substances on guanylate cyclase, guanylate

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cyclase activity in the assay mixture should be measured in the absence of the GTP regenerating system, cyclic GMP and cyclic GMP phosphodiesterase inhibitors. In this communication, we report our purification procedure of human platelet guanylate cyclase and the effect of arachidonic acid peroxide on the purified enzyme which contains virtually no cyclic nucleotide and GTP hydrolytic activities.

MATERIALS AND METHODS

[^{3}H]GTP and cyclic[^{3}H]GMP were purchased from the Radiochemical Centre and cyclic GMP and hemoglobin (human) from Sigma Chemical Co. GTP, creatine phosphate, creatine phosphokinase were obtained from Boehringer Mannheim Co. Arachidonic acid and lipoxidase (soybean) were purchased from P-L Biochemicals Inc. Neutral aluminum oxide was a product of M. Woelm Co. DEAE-cellulose (Whatman, DE52) and agarose gel (Pharmacia, Sepharose 6B) were equilibrated with column buffer prior to use. Arachidonic acid peroxide was prepared by the method described previously (12), and was dispersed in 50 mM Tris-HCl (pH 7.5) by sonication. 0.3 ml of 1 mM arachidonic acid emulsion was incubated with 3 μg of lipoxidase for 10 min at 30°C. This emulsion treated with lipoxidase (peroxide value 1.2 m equivalent/l) was referred to as arachidonic acid peroxide.

Platelet-rich plasma was prepared by low-speed centrifugation of freshly obtained citrated whole blood (13). Platelets were isolated from the platelet-rich plasma by centrifugation and then stored at -70°C until use. Platelet homogenates were prepared from freeze-thaw platelets in 50 mM Tris-acetate buffer (pH 6.0) containing 20 mM 2-mercaptoethanol and 0.1 mM EGTA (ethylene glycol-bis-(β-amino-ethyl ether)N',N'-tetraacetic acid) (Buffer A) using homogenizer tube with tight Teflon pestle. The homogenates were centrifuged for 60 min at 105,000 x g.

Activity of guanylate cyclase was determined by the method of Nakazawa et al. (14). The assay mixture contained 1 mM [^{3}H]GTP (5 Ci/mole), 100 μM cyclic GMP, 15 mM creatine phosphate, 40 μg of creatine kinase, 3 μM $MnCl_2$, 100 μg of bovine serum albumin, 50 mM Tris-HCl (pH 7.7), arachidonic acid peroxide (0.12 m equivalent/l), 3 μg of hemoglobin which enhances the stimulation of guanylate cyclase by the peroxide (12), and an appropriate amount of the enzyme in a total volume of 0.2 ml. The reaction was started by the addition of enzyme. After the mixture was incubated for 20 min at 30°C, the reaction was stopped by heating for 2 min in a boiling bath, following the addition of 1 M HCl (40 μl). Cyclic GMP formed enzymatically was isolated by the serial use of neutral aluminum oxide/AG1-X2 column.

Cyclic GMP phosphodiesterase activity was measured by the method of Hidaka and Shibuya (15). Protein was measured by the method of Lowry et al. (16). Sepharose 6B column was calibrated with Blue Dextran 2000, catalase, aldolase and bovine serum albumin.

RESULTS

Guanylate cyclase activity was purified by DEAE-cellulose and Sepharose 6B chromatography (Fig. 1). This preparation was stored at 0°C and referred to as the purified guanylate cyclase. A summary of the enzyme purification

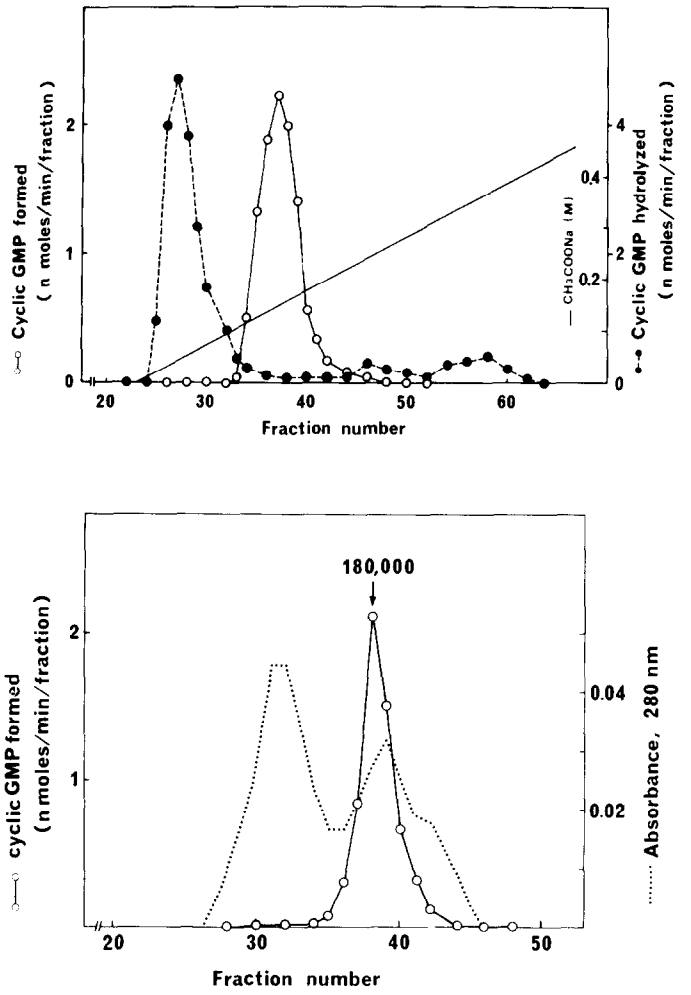

Fig. 1 Elution profiles of guanylate cyclase from columns of DEAE-cellulose (upper) and Sepharose 6B (bottom). (Upper): DEAE-cellulose chromatography; 8 ml of 105,000 x g supernatant of human platelet homogenate was applied to a column (1.5 x 20 cm) of DEAE-cellulose previously equilibrated with Buffer A. The column was washed with approximately 5 volumes of the equilibrating buffer followed by 300 ml of a linear gradient of sodium acetate from 0 to 0.5 M in the same buffer at a flow rate of 30 ml/h and 5 ml fraction was collected. Fractions containing enzymic activity were collected and brought to 30% saturation with solid ammonium sulfate. The precipitate was discarded and ammonium sulfate was added to the supernatant to make a final solution of 50% saturation. The resulting precipitate was then collected by centrifugation, dissolved in 2 ml of Buffer A and applied to a Sepharose 6B column (1.5 x 90cm) ○—○ guanylate cyclase activity and ●---● cyclic GMP hydrolytic activity. (Bottom): Gel filtration on Sepharose 6B; Elution was performed with 50 mM Tris-HCl buffer (pH 7.4) containing 1 M KCl and 20 mM 2-mercaptoethanol and flow rate was 10 ml/hr. Fractions containing guanylate cyclase activity peak were pooled and referred to as the purified enzyme. ○—○ guanylate cyclase activity and ------- protein concentration (OD_{280} mμ).

Table 1. Purification of guanylate cyclase from human platelets

Fraction	Volume	Protein	Activity[a]	Specific activity	Purification
	ml	mg	nmole/min	nmole/min/mg	
Homogenate	8.7	147	36.93	0.25	1
105,000 x g supernatant	8.0	96	30.19	0.32	1.3
DEAE-cellulose eluate	25.0	3.5	10.81	3.09	12.3
Ammonium sulfate fraction	1.6	1.3	5.99	4.61	18.4
Sepharose 6B eluate	10.0	0.17	4.43	26.1	104

[a] Guanylate cyclase activity was measured at its optimal condition; the reaction mixture contained 1 mM [^{3}H]GTP, 2.5 mM cyclic GMP, 15 mM creatine phosphate, 40 µg of creatine kinase, 3 mM $MnCl_2$, 50 mM Tris-HCl (pH 7.7), arachidonic acid peroxide (0.12 m equivalent/l), 3 µg of hemoglobin and an appropriate amount of the enzyme.

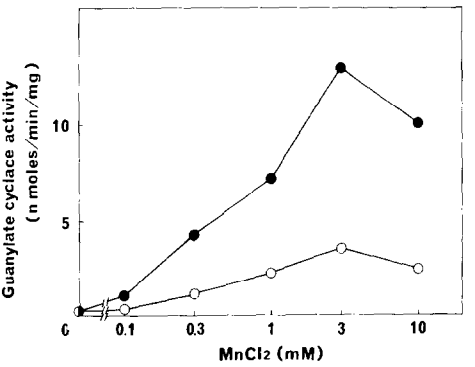

Fig. 2 Effect of arachidonic acid peroxide on Mn^{2+}-requirement of the purified guanylate cyclase. Reaction mixture contained 50 mM Tris-HCl (pH 7.7), 1 mM [^{3}H]GTP; 100 µg of bovine serum albumin. Two mM dithiothreitol was included in the absence of arachidonic acid peroxide (○) and 3 µg of hemoglobin was added in the presence of arachidonic acid peroxide (0.12 m equivalent/l) (●).

is shown in Table 1. All following experiments were carried out with the purified enzyme. The guanylate cyclase was eluted from the column in a volume consistent with a molecular weight of approximately 180,000.

Table 2 shows the requirements of the purified cyclase preparation for cyclic GMP formation. The enzyme activity was dependent on Mn^{2+} with optimal rate at 3 mM (Table 2, Fig. 2) and arachidonic acid peroxide did not alter the concentration of Mn^{2+} required for optimal activity. The activity with Mg^{2+} was less than 20% of that observed with Mn^{2+}. Stimulation by Ca^{2+} was slight. The activity without cyclic GMP was about 95% of that obtained in the presence of 100 µM cyclic GMP while the activity without GTP regenerating system about 90% of that obtained in the presence of GTP system. As 85% of guanylate cyclase activity at optimal conditions was observed in the absence of the cyclic GMP and GTP regenerating system, the following experiments were carried out without inclusion of these systems. The presence and absence of arachidonic acid peroxide produced no essential difference in requirements of the purified enzyme for cyclic GMP formation (Table 2).

Effects of arachidonic acid peroxide on kinetic analysis of guanylate

Table 2. Requirement of the purified enzyme for cyclic GMP formation.

Reaction mixture[a]	Guanylate cyclase activity			
	Without arachidonic acid peroxide		With arachidonic acid peroxide	
	nmole/min/mg	%	nmole/min/mg	%
Complete	3.47	100	13.8	100
Minus $MnCl_2$	0.02	0.5	0.39	2.8
Minus $MnCl_2$, plus $MgCl_2$ (3 mM)	0.38	11	2.76	20
Plus $CaCl_2$ (1 mM)	3.89	112	15.9	115
Minus cyclic GMP	3.26	94	13.2	96
Minus GTP regenerating system	3.19	92	12.4	90
Minus cyclic GMP, minus GTP regenerating system	2.98	86	11.9	86

[a] The complete reaction mixture contained 50 mM Tris-HCl (pH 7.7), 1 mM [^{3}H]GTP, 3 mM $MnCl_2$, 100 μM cyclic GMP, GTP regenerating system, 100 μg of bovine serum albumin and the purified enzyme preparation. Two mM dithiothreitol was included in the absence of arachidonic acid peroxide and 3 μg of hemoglobin was added in the presence of arachidonic acid peroxide (0.12 m equivalent/l).

cyclase were then studied. Arachidonic acid peroxide caused about 4-fold increase in maximal velocity but no change in Km for GTP (Fig. 3). The Km value was 95 ± 3 μM in the presence or absence of peroxide. Table 3 shows the effect of various compounds on guanylate cyclase. A high concentration (above 1 mM) of cyclic GMP stimulated guanylate cyclase and such could not be attributed to protection of the cyclic GMP degradation as over 90% of cyclic [^{3}H]GMP added at the start of incubation was recovered in the reaction mixture. ATP inhibited guanylate cyclase activity to the same extent in the presence and absence of the peroxide.

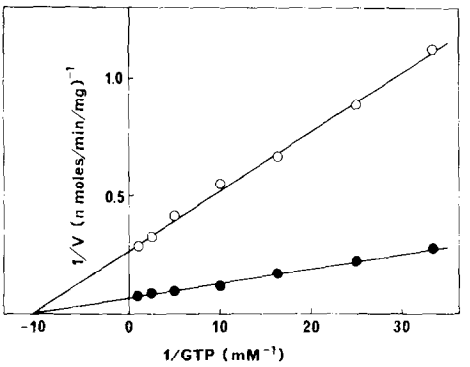

Fig. 3 Lineweaver-Burk plots of cyclic GMP formation by the purified human platelet guanylate cyclase in the absence (O) and presence (●) of arachidonic acid peroxide (0.12 m equivalent/l) and 3 μg hemoglobin. Control reaction mixture (without arachidonic acid peroxide) contained 2 mM dithiothreitol instead of the peroxide and hemoglobin. The substrate (GTP) concentration was varied from 0.02 to 1 mM with 50 mM Tris-HCl (pH 7.7), 3 mM $MnCl_2$ and 100 μg bovine serum albumin.

DISCUSSION

Stimulation of guanylate cyclase by arachidonic acid peroxide was attributed to oxidation of enzyme protein and guanylate cyclase activity in the crude preparation from human platelets increased during incubation and during storage for 2-3 days at 4°C (12). Effect of arachidonic acid peroxide on the enzyme stored 2-3 days at 4°C was considerably less compared to that of the freshly prepared enzyme. Böhme et al. (17) reported that stimulation of guanylate cyclase during incubation was prevented by the addition of dithiothreitol and suggested that this was due to the oxidation of a residue in enzyme protein. We used a buffer containing an extremely high concentration of 2-mercaptoethanol (20 mM) for purification and added 2 mM dithiothreitol to the assay mixture. Guanylate cyclase activity in the purified preparation was linear during incubation and stimulated 4-fold by arachidonic acid peroxide even after stored for 2-3 days at 4°C under this condition (20 mM 2-mercaptoethanol).

As shown in Table 3, only a 15% decrease in the purified guanylate cyclase

Table 3. Effect of nucleotides on purified guanylate cyclase.

Additions	Guanylate cyclase activity[a]			
	Without arachidonic acid peroxide		With arachidonic acid peroxide	
	nmole/min/mg	%	nmole/min/mg	%
None	3.10	100	13.0	100
Cyclic GMP 1 mM	4.55	147	17.9	138
Cyclic GMP 2.5 mM	5.58	180	20.8	160
ATP 0.1 mM	2.73	88	10.5	81
ATP 1 mM	0.65	21	3.51	27

[a] Guanylate cyclase from Sepharose 6B was used and the reaction mixture contained 50 mM Tris-HCl (pH 7.7), 1 mM [^{3}H]GTP, 3 mM $MnCl_2$, 100 μg of bovine serum albumin. Two mM dithiothreitol was included in the absence of arachidonic acid peroxide and 3 μg hemoglobin was added in the presence of arachidonic acid peroxide (0.12 m equivalent/l).

activity was observed in the assay mixture when the GTP regenerating system and cyclic GMP were omitted. Properties of guanylate cyclase were then investigated using this purified preparation. A low concentration (100 μM) of cyclic GMP did not affect guanylate cyclase (Table 2) but a high concentration (2.5 mM) of cyclic GMP stimulated the activities 1.6- to 1.8-fold and this stimulation was not due to the pretection for cyclic GMP degradation (Table 3). It should be noted that guanylate cyclase activity determined in the presence of a high concentration of cyclic GMP is not a true basal activity. Km value and optimal Mn^{2+} concentration obtained in this study roughly agree with values reported by Glass et al. (11) who used 105,000 x g supernatant as an enzyme preparation.

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