Factors Affecting the Activity of Guanylate Cyclase in Lysates of Human Blood Platelets

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1. Under optimal ionic conditions (4 mM-MnCl₂) the specific activity of guanylate cyclase in fresh platelet lysates was about 10 nmol of cyclic GMP formed/20 min per mg of protein at 30°C. Activity was 15% of optimum with 10 mM-MgCl₂ and negligible with 4 mM-CaCl₂. Synergism between MnCl₂ and MgCl₂ or CaCl₂ was observed when [MnCl₂] ≤ [GTP]. 2. Lower than optimal specific activities were obtained in assays containing large volumes of platelet lysate, owing to the presence of inhibitory factors that could be removed by ultrafiltration. Adenine nucleotides accounted for less than 50% of the inhibitory activity. 3. Preincubation of lysate for 1 h at 30°C increased the specific activity of platelet guanylate cyclase by about 2-fold. 4. Lubrol PX (1%, w/v) stimulated guanylate cyclase activity by 3–5-fold before preincubation and by about 2-fold after preincubation. Triton X-100 was much less effective. 5. Dithiothreitol inhibited the guanylate cyclase activity of untreated, preincubated and Lubrol PX-treated lysates and prevented activation by preincubation provided that it was added beforehand. 6. Oleate stimulated guanylate cyclase activity 3–4-fold and arachidonate 2–3-fold, whereas palmitate was almost inactive. Pretreatment of lysate with indomethacin did not inhibit this effect of arachidonate. Oleate and arachidonate caused marked stimulation of guanylate cyclase in preincubated lysate, but inhibited the enzyme in Lubrol PX-treated lysate. 7. NaN₃ (10 mM) increased guanylate cyclase activity by up to 7-fold; this effect was both time- and temperature-dependent. NaN₃ did not further activate the enzyme in Lubrol PX-treated lysate. 8. The results indicated that preincubation, Lubrol PX, fatty acids and NaN₃ activated platelet guanylate cyclase by different mechanisms. 9. Platelet particulate fractions contained no guanylate cyclase activity detectable in the presence or absence of Lubrol PX that could not be accounted for by contaminating soluble enzyme, suggesting that physiological aggregating agents may increase cyclic GMP in intact platelets through the effects of intermediary factors. The activated and inhibited states of the enzyme described in the present paper may be relevant to the actions of these factors.

A number of agents that can induce platelet aggregation and the secretion of platelet granule contents in the so-called release reaction have also been reported to increase the intracellular concentrations of cyclic GMP in platelets (White *et al.*, 1973; Haslam & McClenaghan, 1974; Haslam, 1975; Jakobs *et al.*, 1975; Chiang *et al.*, 1975; Davies *et al.*, 1976; Glass *et al.*, 1977b). Although the role of cyclic GMP in platelet function is still uncertain, the observed increases in cyclic GMP appear to correlate with platelet aggregation rather than with the release reaction (Davies *et al.*, 1976; Haslam, 1977; Haslam *et al.*, 1978). The mechanism by which physiological stimuli increase cyclic GMP formation in intact platelets or in other cells is unknown. Agents that promote cyclic GMP formation in intact cells have in general not been found to stimulate the activity of guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] in broken-cell preparations

(Goldberg & Haddox, 1977). These observations, together with the presence of soluble as well as membrane-bound guanylate cyclase in most cells (Kimura & Murad, 1975), have led to suggestions that guanylate cyclase may be activated by indirect mechanisms in intact cells (Schultz *et al.*, 1973; Goldberg & Haddox, 1977). The platelet is an excellent system in which to study this problem, as platelet guanylate cyclase has been shown to be a predominantly soluble enzyme with a specific activity higher than that in almost all other mammalian tissues (Böhme *et al.*, 1974; Barber, 1976; Rodan & Feinstein, 1976; Glass *et al.*, 1977a). Although other workers have investigated specific aspects of platelet guanylate cyclase, notably its bivalent-cation requirements (Böhme *et al.*, 1974; Rodan & Feinstein, 1976) and activation by certain fatty acids (Barber, 1976; Glass *et al.*, 1977a; Hidaka & Asano, 1977), we have studied the effects and interactions of a wide range of

factors that were found to affect the activity of the enzyme in platelet lysates with a view to defining possible mechanisms of activation *in vivo*. Some of these results have been reported briefly elsewhere (Adams & Haslam, 1978).

Experimental

Materials

[8-³H]GTP (10Ci/mmol), cyclic [8-¹⁴C]GMP (60mCi/mmol) and cyclic [8-³H]GMP (13Ci/mmol) were obtained from Amersham Corp., Oakville, Ont., Canada. [8-³H]GTP was purified by t.l.c. in two dimensions on cellulose, by using the solvents described by Haslam & McClenaghan (1974). The following reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.: protein standard solution, crystalline bovine serum albumin, creatine kinase (150 units/mg of protein) and phosphocreatine, cyclic nucleotide phosphodiesterase (0.27 unit/mg), other enzymes used in assays, all nucleotides, aspirin, sodium salicylate, indomethacin, 5-hydroxytryptamine, (–)-adrenaline, arachidonic acid, NaN₃, dithiothreitol, glutathione, *N*-ethylmaleimide, Lubrol PX, Triton X-100, Trizma (Tris) base, triethanolamine hydrochloride, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, and neutral alumina (WN-3). Arachidonic acid was purified as described by Davies *et al.* (1976). 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Cellulose powder for t.l.c. (MN 300 HR) was obtained from Brinkman Instruments (Canada), Toronto, Ont., Canada, and anion-exchange resin (AG 1-X2; 200–400 mesh; Cl[–] form) from Bio-Rad Laboratories, Richmond, CA, U.S.A. Ultrafiltration membrane cones (Centriflo, 224-CF-50) were purchased from Amicon Co., Lexington, MA, U.S.A., and oleic acid and palmitic acid from the Hormel Institute, Austin, MN, U.S.A. Liquid-scintillation 'cocktail' (Quantafluor) was obtained from Mallinckrodt, St. Louis, MO, U.S.A. Other reagents were of analytical grade.

Methods

Enzyme preparations. Platelets were isolated and washed as described previously (Haslam & Rosson, 1972) and were finally resuspended in 150mM-Tris, adjusted to pH 7.4 with HCl, to give 50–100mg wet wt. of platelets/ml (approx. 3.5–7.0mg of protein/ml). After 30min at 0°C, the suspension was frozen in a solid-CO₂/acetone bath and was then gently shaken in a water bath at 37°C until just completely thawed. The freeze-thaw cycle was repeated once and the platelet lysate was usually stored at 0°C for up to 2h before use. In some experiments, part of the lysate

was preincubated for 1h at 30°C and then stored at 0°C. In other experiments, lysate was ultrafiltered in Centriflo membrane cones that had been soaked in water for 1h. In the latter case, up to 2ml was centrifuged at 1000g for 1h at 4°C. The volume of the ultrafiltrate was measured (70–90% of that of the lysate) and the concentrate was resuspended in an equivalent volume of 150mM-Tris/HCl, pH 7.4, and stored at 0°C. Ultrafiltration resulted in a loss of about 20% of lysate protein on the ultrafiltration membrane.

Guanylate cyclase assay. Assay mixtures (250μl) contained (final concentrations): 1mM-[8-³H]GTP (2mCi/mmol), 1mM-3-isobutyl-1-methylxanthine, 4mM-cyclic GMP, 1mg of bovine serum albumin/ml, 4mM-MnCl₂ and 60mM-Tris/HCl, pH 7.4. Phosphocreatine (5mM) and creatine kinase (20 units/ml) were included unless otherwise indicated. Assays were started by the addition of 10–100μl of enzyme preparation to freshly mixed reagents at 30°C. Incubations were continued for 20min at 30°C and were stopped by the addition of 100μl of a solution containing 0.001μCi of cyclic [8-¹⁴C]-GMP (60mCi/mmol) and 50mM-EDTA (adjusted to pH 7.4 with NaHCO₃), followed by boiling for 3min. The samples were finally transferred to an ice bath and 50mM-Tris/HCl, pH 7.6, was added to give a total volume of 1ml. Denatured protein was removed by centrifugation.

Cyclic [³H]GMP was isolated by a modification of the method of White & Zenser (1971). Sample supernatants were applied to columns (internal diameter 6mm) containing 1g of neutral alumina, which had been washed with 50mM-Tris/HCl, pH 7.6. Cyclic GMP was then eluted with 50mM-Tris/HCl, pH 7.6; the first 2ml from each column was discarded and the next 3ml was collected into liquid-scintillation vials and freeze-dried. The residues were dissolved in 1ml of water, mixed with 7.5ml of Quantafluor, and counted for ³H and ¹⁴C radioactivity as described by Haslam & McClenaghan (1974). Recovery of added cyclic [8-¹⁴C]GMP in the fraction collected ranged from 40 to 60%. Zero-incubation-time blanks were included in each experiment and amounted to 100–200d.p.m. of ³H (about 5% of the total d.p.m. of cyclic [³H]GMP recovered during a typical assay). This blank was subtracted before correction for recovery. Similar blank values were obtained from samples without enzyme which were not boiled, indicating that non-enzymic formation of cyclic [³H]GMP during boiling (Kimura & Murad, 1974a) did not occur. The successful use of [8-³H]GTP in this assay was dependent on prior purification of the substrate, and the removal by freeze-drying of small amounts of ³H₂O formed during boiling of the samples. Individual assays were performed in triplicate. Mean values are reported. Standard errors were almost always less than ±5% of the

mean. The effects reported were observed with lysates from at least three separate platelet preparations.

Radiochemical purity of cyclic [^3H]GMP isolated from guanylate cyclase assays. Pooled eluates from identical samples applied to alumina columns were chromatographed on anion-exchange resin (Dowex AG 1-X2). Cyclic GMP was eluted as described by Haslam & McClenaghan (1974) and the eluate was freeze-dried. The residue was taken up in water, a sample was counted for ^3H and ^{14}C radioactivity and another sample was incubated for 60 min at 30°C in a reaction mixture (120 μl) containing 0.05 unit of cyclic nucleotide phosphodiesterase, 4 mg of bovine serum albumin/ml, 4 mM- MgSO_4 , 0.25 mM-EDTA and 10 mM-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (adjusted to pH 7.5 with NaOH). The reaction mixture was boiled and centrifuged, and the supernatant was chromatographed on cellulose as described by Haslam & McClenaghan (1974). Product 5'-GMP was eluted and counted for ^3H and ^{14}C radioactivity. The ratio of ^3H to ^{14}C in this 5'-GMP was the same as in freeze-dried eluates from the columns containing either alumina or anion-exchange resin, indicating that the standard assay procedure isolated radiochemically pure cyclic [^3H]GMP.

Measurement of cyclic GMP phosphodiesterase and guanosine triphosphatase activities present in guanylate cyclase assays. The activity of cyclic GMP phosphodiesterase remaining under guanylate cyclase assay conditions was determined by substituting unlabelled GTP for [^3H]GTP and adding cyclic [^3H]GMP (0.025 μCi) to the assay mixture. Labelled cyclic GMP was isolated as usual and the percentage of cyclic [^3H]GMP broken down was shown to be less than 7% in 20 min incubations with the highest enzyme concentration used in this study.

To measure GTP breakdown, guanylate cyclase assays were carried out as usual, but the reactions were stopped by the addition of 100 μl of 50 mM-EDTA, pH 7.4, followed by boiling for 3 min. After centrifugation, 10 μl of the supernatant was mixed with 0.1 μmol each of GTP and of GDP and chromatographed on cellulose in two dimensions (Haslam & McClenaghan, 1974). The areas corresponding to GTP and GDP were eluted with 2 ml of water, the extinction values at 253 nm were measured and fractions of this material were counted for ^3H radioactivity. Guanosine triphosphatase activity was expressed as a percentage of the [^3H]GTP added and amounted to about 1%/min with 300 μg of platelet protein in an assay tube. Therefore, in most experiments in which more protein was used or in which incubations were continued for longer than 20 min, phosphocreatine and creatine kinase, which prevented GTP breakdown, were included in the assay mixtures. However, under all of these conditions the presence of a GTP-regenerating system had no

significant effects on the accumulation of cyclic [^3H]GMP.

Other methods. Fatty acids were dissolved in ethanol, neutralized with Na_2CO_3 and diluted with water to a final concentration of 20 mM [containing 10% (v/v) ethanol]. In experiments with fatty acids, the final concentration of ethanol in the assays was adjusted to 0.5%, which has no effect on guanylate cyclase activity. Lactate dehydrogenase activity in platelet fractions was determined by the method of Bergmeyer *et al.* (1965). Protein was assayed by the method of Lowry *et al.* (1951), with a protein standard solution containing 5% (w/v) human albumin and 3% (w/v) human globulin. Tris/HCl blanks were subtracted (Ji, 1973).

Results

Bivalent-cation requirements

Maximum guanylate cyclase activity of the platelet lysate was observed in the presence of 4 mM- MnCl_2 and 1 mM-GTP. In the absence of added MnCl_2 , only 15% of maximum activity was expressed with MgCl_2 at an optimal concentration of 10 mM, whereas almost no activity was measurable with 4 mM- CaCl_2 (Table 1). However, in the presence of lower concentrations of MnCl_2 (0.1 or 1 mM) together with either MgCl_2 (10 mM) or CaCl_2 (4 mM), a synergistic stimulation of guanylate cyclase activity was observed (Table 1). The lowest concentration of CaCl_2 at which a synergistic effect could be detected was 0.4 mM.

Inhibition of guanylate cyclase activity by factors present in platelet lysate

The average specific activity of guanylate cyclase measured with 10 or 20 μl of lysate prepared from platelet suspensions containing 100 mg wet wt. of platelets/ml was about 10 nmol of cyclic GMP formed/20 min per mg of protein. However, when 50 or 100 μl of lysate was assayed, the specific activity was decreased on average by 30% and 60% respectively (Figs. 1a and 1b). This effect was not due to either guanosine triphosphatase or cyclic GMP phosphodiesterase activity (see under 'Methods'). Ultrafiltration of the lysate caused highly significant increases in the specific activity of guanylate cyclase in assays of 50 and 100 μl of enzyme, whereas no effect was observed with smaller volumes (Fig. 1a). Addition of the ultrafiltrate, which contained no measurable protein, to the ultrafiltered lysate or to the untreated lysate demonstrated the presence of low-molecular-weight inhibitory factors. The guanylate cyclase activity of 50 μl of lysate was decreased by an average of 40% by 50 μl of the ultrafiltrate

Table 1. *Effects of bivalent cations on the guanylate cyclase activity of platelet lysate*

Preparation of the platelet lysate and assay of guanylate cyclase activity were carried out as described under 'Methods'. Each assay contained 175 μ g of platelet protein.

Concn. of bivalent cation (mM)			Guanylate cyclase activity	
MnCl ₂	MgCl ₂	CaCl ₂	(nmol of cyclic GMP/20 min per mg of protein)	(% of activity with 4 mM-MnCl ₂)
4.0	0	0	8.40	100
4.0	10.0	0	9.53	113
1.0	0	0	3.03	36
1.0	10.0	0	6.28	75
1.0	0	4.0	5.07	60
1.0	10.0	4.0	7.42	88
0.1	0	0	0.14	2
0.1	10.0	0	1.90	23
0.1	0	4.0	0.73	9
0.1	10.0	4.0	1.04	12
0	10.0	0	1.26	15
0	0	4.0	0.16	2
0	10.0	4.0	0.83	10

Fig. 1. *Effects of ultrafiltration (a) and of preincubation (b) of platelet lysate on the specific activity of guanylate cyclase measured with different volumes of platelet lysate in the assay*

Platelet lysates were preincubated or ultrafiltered as described under 'Methods'. Increasing volumes were assayed by the addition of 10, 20, 50 and 100 μ l to the assay mixtures, which were incubated for 20 min. In some of these experiments, a GTP-regenerating system was not included in the assay mixture. Although protein concentrations in the lysates varied in different experiments as indicated below, for statistical purposes paired data were grouped according to the volume of lysate added to the assays, and the significance of the effects of preincubation or ultrafiltration were determined by paired *t* tests: * $2P < 0.01$, ** $2P < 0.001$. (a) ●, Untreated lysate; ■, ultrafiltered lysate; results are from nine experiments in which the untreated lysates contained 5.5 ± 0.8 mg of protein/ml (mean $\pm$ s.e.m.) and the ultrafiltered lysates contained 4.3 ± 0.6 mg of protein/ml (mean $\pm$ s.e.m.). (b) ●, Untreated lysate; ▲, preincubated lysate; results are from 12 experiments in which the lysates contained 6.3 ± 0.9 mg of protein/ml (mean $\pm$ s.e.m.).

(Table 2). Although treatment of the ultrafiltrate with charcoal completely removed its inhibitory activity, only up to half of the observed inhibitory

effect could be accounted for by platelet adenine nucleotides (Table 2, Fig. 2). ATP and ADP inhibited guanylate cyclase activity to similar extents.

Table 2. Inhibition of the guanylate cyclase activity of platelet lysate by lysate ultrafiltrate

In each experiment ultrafiltrate was prepared from platelet lysate as described under 'Methods'. The A_{260} of the ultrafiltrate was measured and the adenine nucleotide concentration added to the assay mixtures calculated. The expected inhibition of guanylate cyclase activity by an equivalent concentration of ATP was calculated from Fig. 2. In each experiment 50 μ l samples were assayed; protein per assay was: (1) 210 μ g; (2) 350 μ g; (3) 260 μ g.

Expt. no.	Volume of ultrafiltrate added (μ l)	Final concn. of added nucleotides (mM)	Guanylate cyclase activity		Inhibition of activity predicted from Fig. 2 (%)
			(nmol of cyclic GMP/20 min per mg of protein)	Inhibition by ultrafiltrate (%)	
1	0	—	6.14	0	—
	50	0.06	3.67	40	16
2	0	—	3.60	0	—
	50	0.05	2.03	44	15
3	0	—	4.90	0	—
	50	0.05	3.48	29	15

Fig. 2. Inhibition of platelet guanylate cyclase by ATP. Assays contained 355 μ g of platelet protein with or without added ATP.

the volume of lysate assayed; average increases in guanylate cyclase activity of 50, 100 and 200% were seen with 20, 50 and 100 μ l respectively of preincubated lysate (Fig. 1*b*). This increase in guanylate cyclase activity of 50 μ l of lysate after preincubation was only partly attributable to decreased inhibition by ultrafilterable factors, because ultrafiltrate from preincubated lysate retained a large proportion of the original inhibitory activity (59 and 86% respectively, in two experiments). Preincubated lysate was as sensitive to inhibition by ultrafiltrate as untreated lysate, and ultrafiltered lysate showed a substantial increase in guanylate cyclase activity during assay (Table 3).

Effects of non-ionic detergents

Two non-ionic detergents, Triton X-100 and Lubrol PX, were studied; the latter was much more effective at all concentrations tested (Fig. 3). In 20 min assays, optimal activation (3–5-fold) was observed with 1% (w/v) Lubrol PX (44 mM), whether this concentration of detergent was included in the assay mixture (Fig. 3) or added to the lysate before the assay (Table 4). Thus the extent of activation by Lubrol PX depended on the highest concentration to which the enzyme was exposed and not on the final concentration in the assay mixture.

The guanylate cyclase activity of Lubrol PX-treated lysate, unlike that of untreated lysate, did not increase during assay (Table 4). Treatment of preincubated lysate with Lubrol PX did not result in an additive stimulation of enzyme activity; instead, the specific activity of this preparation was either equal to or slightly lower than that of Lubrol PX-treated lysate that had not been preincubated (Tables 4 and 5). Preincubation in the presence of Lubrol PX resulted in a substantial inhibition of guanylate cyclase activity.

Activation of guanylate cyclase by preincubation of lysate

The specific activity of guanylate cyclase of platelet lysate increased during assay (Table 3). The average specific activity of the enzyme during the interval between 20 and 40 min after starting the assay was over 2-fold greater than the value measured during the initial 5 min of incubation. This increase in activity was not dependent on components present in the assay medium, as preincubation of the lysate for 1 h at 30°C also had a stimulatory effect (Table 3). However, the magnitude of this effect depended on

Table 3. *Changes in the guanylate cyclase activities of platelet lysate, preincubated lysate and ultrafiltered lysate during incubation*

Results were pooled from the numbers of experiments listed below. Platelet lysate, preincubated lysate and ultrafiltered lysate were prepared as described under 'Methods'. The mean values $\pm$ S.E.M. for the protein assayed were: untreated lysate, $200 \pm 15 \mu\text{g}$; preincubated lysate, $185 \pm 10 \mu\text{g}$; ultrafiltered lysate, $145 \pm 20 \mu\text{g}$. Incubations were for 5, 20 and 40 min. In some of these experiments a GTP-regenerating system was not included in the assay mixtures. Guanylate cyclase activity is expressed in terms of specific activity determined for three intervals: 0–5 min, 5–20 min and 20–40 min. The ratio of the specific activities (20–40 min/0–5 min) reflects the acceleration of guanylate cyclase activity in each of these preparations. Values for guanylate cyclase activity are the means $\pm$ S.E.M.

Enzyme preparation	No. of experiments	Guanylate cyclase activity (nmol pf cyclic GMP/min per mg of protein)			Ratio of guanylate cyclase activities (20–40 min/0–5 min)
		0–5 min	5–20 min	20–40 min	
Untreated lysate	12	0.28 ± 0.03	0.49 ± 0.02	0.61 ± 0.04	2.18
Preincubated lysate	10	0.64 ± 0.06	0.87 ± 0.09	0.87 ± 0.12	1.36
Ultrafiltered lysate	6	0.48 ± 0.08	0.60 ± 0.06	0.78 ± 0.09	1.63

Fig. 3. *Effects of Lubrol PX and of Triton X-100 on the guanylate cyclase activity of platelet lysate*

Platelet lysate was added to assay mixtures containing Lubrol PX or Triton X-100 at the final concentrations indicated on the abscissa. Each assay contained $330 \mu\text{g}$ of platelet protein. ■, No addition; ●, Lubrol PX; ▲, Triton X-100.

Effect of thiols and of N-ethylmaleimide

Addition of 5 mM-dithiothreitol to platelet lysate inhibited the guanylate cyclase activity of both control and Lubrol PX-treated lysates by about 70% and if present during preincubation of lysate prevented any increase in activity (Table 5). However, dithiothreitol inhibited the activity of enzyme that

had already been preincubated by the same percentage as control enzyme, so that in this case the preincubation effect could still be detected. The guanylate cyclase activity of platelet lysate was almost completely blocked by 10 mM-glutathione or 1 mM-N-ethylmaleimide.

Effects of fatty acids

The effects on inducers of platelet aggregation on the guanylate cyclase activity of lysate were tested. In contrast with 5-hydroxytryptamine ($10 \mu\text{M}$) and (–)-adrenaline ($20 \mu\text{M}$), which were without effect, and with ADP ($100 \mu\text{M}$), which inhibited enzyme activity by about 30% (measured in the absence of phosphocreatine with creatine kinase), arachidonate stimulated the activity of platelet guanylate cyclase. The specificity and physiological relevance of this last effect were studied further.

The guanylate cyclase activity of platelet lysate was stimulated by about 2–3-fold by arachidonate (1 mM) and by about 3–4-fold by oleate (1 mM), whereas palmitate was almost inactive (Table 6). Indomethacin, a fatty acid cyclo-oxygenase inhibitor, had no effect on either the basal or fatty acid-stimulated guanylate cyclase activities; thus the observed effect of arachidonate cannot be attributed to its metabolism to prostaglandin endoperoxides.

The effects of these fatty acids on preincubated or Lubrol PX-treated lysate were also studied. In percentage terms, the guanylate cyclase activity of preincubated lysate was stimulated only slightly less by either arachidonate or oleate than the untreated lysate (Table 6). Thus the actual increase in enzyme activity caused by preincubation of the lysate and subsequent addition of these fatty acids was greater than the sum of the individual treatments (Table 6). Oleate increased the guanylate cyclase activity of preincubated lysate to values far higher than observed on treatment of lysate with Lubrol PX. In contrast,

Table 4. *Effects of Lubrol PX and of NaN_3 on the guanylate cyclase activities of platelet lysate and of preincubated lysate assayed for different periods*

Preparation and preincubation of platelet lysate and assay of guanylate cyclase activity (without a GTP-regenerating system) were carried out as described under 'Methods'. Lubrol PX (1%, w/v) or NaN_3 was added to lysate at 0°C 10min before assay or was preincubated at 30°C as indicated below. Each assay contained 200 μg of platelet protein. Incubations were carried out for 5 or 20min. Guanylate cyclase activity is expressed in terms of specific activity determined for the two sequential intervals 0–5min and 5–20min. The ratio of the specific activities (5–20min/0–5min) reflects the acceleration of guanylate cyclase activity.

Enzyme preparation	Addition to the enzyme preparation	Preincubation at 30°C with addition (h)	Guanylate cyclase activity				Ratio of guanylate cyclase activities (5–20min/0–5min)
			0–5 min		5–20min		
			(nmol of cyclic GMP/min per mg of protein)	(% of control activity)	(nmol of cyclic GMP/min per mg of protein)	(% of control activity)	
Lysate	None	—	0.14	100	0.30	100	2.14
	Lubrol PX	—	0.92	657	1.02	340	1.11
	NaN ₃	—	0.43	307	2.03	677	4.72
Preincubated lysate	None	0	0.34	100	0.44	100	1.29
	Lubrol PX	0	0.86	253	0.88	200	1.02
	Lubrol PX	1	0.18	53	0.17	39	0.94
	NaN ₃	0	0.88	259	2.06	468	2.34
	NaN ₃	1	1.93	258	2.36	536	1.22

Table 5. *Effect of dithiothreitol treatment of platelet lysate on the guanylate cyclase activities observed before and after preincubation*

Preparation of the platelet lysate and assay of guanylate cyclase activity (without a GTP-regenerating system) were carried out as described under 'Methods'. Platelet lysate was preincubated for 80min at 30°C with and without 5mm-dithiothreitol. Guanylate cyclase activity was assayed before and after preincubation in both the presence and absence of Lubrol PX added to the assay mixture at a final concentration of 1% (w/v). Each assay contained 170 μg of platelet protein.

Enzyme preparation	Addition to the assay	Guanylate cyclase activity (nmol of cyclic GMP/10min per mg of protein)		Ratio of guanylate cyclase activities (after/before preincubation)
		Before preincubation	After preincubation	
Lysate	None	8.64	14.41	1.67
	Lubrol PX	39.83	42.00	1.05
Dithiothreitol-treated lysate	None	2.49	2.85	1.14
	Lubrol PX	9.97	9.26	0.93

all three fatty acids exerted an inhibitory effect on the activity of Lubrol PX-treated lysate. Arachidonate was the most effective; at 1mM concentration it decreased the enzyme activity below that of untreated lysate in the absence of arachidonate.

Effects of NaN_3

NaN_3 , added to platelet lysate at 0°C at an optimal concentration of 10mM, stimulated guanylate cyclase activity by up to about 7-fold (Table 4). When NaN_3 was added to the lysate immediately before assay, the guanylate cyclase activity observed during the first 5min of assay was much less than observed subsequently (Table 4). This acceleration of activity during assay was greatly decreased when NaN_3 was added to a lysate that had already been

preincubated and was decreased even more when the lysate was preincubated in the presence of NaN_3 . NaN_3 did not further activate guanylate cyclase in Lubrol PX-treated lysate, although the final enzyme activity in the presence of NaN_3 alone was considerably greater than observed with Lubrol PX alone.

Subcellular distribution of platelet guanylate cyclase

The apparent subcellular distribution of platelet guanylate cyclase activity varied with the osmolarity of the lysis medium. As indicated in Table 7, between 60 and 75% of the total activity was found in the 100000g supernatant fraction when the cells were lysed by freezing and thawing in an iso-osmotic medium (150mM-Tris/HCl, pH7.4). Under these conditions, the specific activity of guanylate cyclase

Table 6. *Effects of fatty acids on the guanylate cyclase activities of untreated lysate, indomethacin-treated lysate, preincubated lysate and Lubrol PX-treated lysate*

Portions of platelet lysate were either stored at 0°C until used, incubated with 20 μ M-indomethacin for 5 min at 30°C, preincubated for 1 h at 30°C or exposed to 1% (w/v) Lubrol PX for 10 min at 0°C. Each assay contained 180 μ g of platelet protein. Fatty acids were added to the assays at the final concentrations indicated.

Enzyme preparation	Fatty acid	Concn. (mM)	Guanylate cyclase activity	
			(nmol of cyclic GMP/ 20min per mg of protein)	(% of control activity)
Untreated lysate	None	—	7.5	100
	Palmitate	0.1	8.4	112
		1.0	8.7	116
	Oleate	0.1	13.0	173
		1.0	25.3	337
	Arachidonate	0.1	12.4	165
1.0		15.6	208	
Indomethacin-treated lysate	None	—	7.6	100
	Palmitate	0.1	8.6	113
		1.0	8.4	111
	Oleate	0.1	13.8	182
		1.0	21.6	284
	Arachidonate	0.1	12.3	162
1.0		16.0	211	
Preincubated lysate	None	—	12.0	100
	Palmitate	0.1	12.3	103
		1.0	13.3	111
	Oleate	0.1	19.3	161
		1.0	37.0	308
	Arachidonate	0.1	18.5	154
1.0		22.3	186	
Lubrol PX-treated lysate	None	—	21.7	100
	Palmitate	0.1	18.3	84
		1.0	15.5	71
	Oleate	0.1	20.3	94
		1.0	13.5	62
	Arachidonate	0.1	14.1	65
1.0		5.6	26	

in the supernatant fraction was somewhat greater than in the particulate fraction. However, when the platelets were frozen and thawed in a hypo-osmotic medium (60mM-Tris/HCl, pH 7.4) about 90% of the enzyme was soluble, and the specific activity of the supernatant was about 8-fold that of the particulate fraction. The distribution of guanylate cyclase activity closely paralleled that of the soluble enzyme marker, lactate dehydrogenase, in both iso-osmotic and hypo-osmotic preparations (Table 7). Treatment of the supernatant and the particulate fractions prepared from either iso-osmotic or hypo-osmotic lysates with Lubrol PX (1%, w/v) did not change the relative distribution of guanylate cyclase activity (Table 7), indicating the absence of latent particle-bound enzyme. These results show that the only particle-bound guanylate cyclase found was due to contamination of particulate fractions with soluble enzyme.

Discussion

Factors affecting the basal activity of platelet guanylate cyclase

The average basal guanylate cyclase activity of platelet lysate, assayed under optimal ionic conditions (4mM-MnCl₂), was approx. 10nmol of cyclic GMP formed/20min per mg of protein at 30°C. This value agrees reasonably well with those reported for platelet guanylate cyclase by other workers (Böhme *et al.*, 1974; Barber, 1976; Glass *et al.*, 1977a), when allowance is made for the higher temperature (37°C) used by others and the presence of dithiothreitol in the assay medium in one study (Glass *et al.*, 1977a). Thus the results confirm that the specific activity of platelet guanylate cyclase is substantially higher than that of any other mammalian cells or tissues studied.

The dependence of platelet guanylate cyclase on

Table 7. *Effect of freezing and thawing of platelets suspended in iso-osmotic and hypo-osmotic media on the subcellular distribution of guanylate cyclase analysed in the presence and absence of Lubrol PX*

Platelets were washed as described under 'Methods' and resuspended in either iso-osmotic medium (150 mM-Tris/HCl, pH 7.4) or hypo-osmotic medium (60 mM-Tris/HCl, pH 7.4) and then lysed by freezing and thawing twice. The lysates were centrifuged at 100000g for 2 h at 4°C and each particulate fraction was resuspended to the volume of the corresponding supernatant with the original buffer. A portion of the supernatant and particulate fractions from both lysate preparations was treated with Lubrol PX. In each case, 50 µl samples were assayed; protein per assay was: iso-osmotic supernatant fraction, 110 µg; iso-osmotic particulate fraction, 110 µg; hypo-osmotic supernatant fraction, 130 µg; hypo-osmotic particulate fraction, 100 µg. The final buffer concentration in the assay mixtures was adjusted to 60 mM in all cases. Guanylate cyclase was assayed in the absence of a GTP-regenerating system. Lactate dehydrogenase activities were assayed as described under 'Methods'. 'Total activity' refers to the sum of the activities in the supernatant and particulate fractions.

Lysis medium	Enzyme preparation	Concn. of Lubrol PX (% w/v)	Guanylate cyclase activity		Distribution of lactate dehydrogenase (% of total activity)
			(nmol of cyclic GMP/20 min per mg of protein)	(% of total activity)	
Iso-osmotic	Supernatant	0	5.22	61	67
		1.0	16.15	67	—
	Particulate	0	3.25	39	33
		1.0	7.82	33	—
Hypo-osmotic	Supernatant	0	5.16	92	89
		1.0	18.34	91	—
	Particulate	0	0.60	8	11
		1.0	2.56	9	—

free Mn^{2+} (i.e. $MnCl_2$ in excess of GTP) for the expression of maximum basal activity has also been reported by others (Böhme *et al.*, 1974; Rodan & Feinstein, 1976; Glass *et al.*, 1977a). Our results also show that neither $MgCl_2$ nor $CaCl_2$ alone could effectively substitute for $MnCl_2$; in contrast, Böhme *et al.* (1974) reported that the activity with high concentrations of $MgCl_2$ (10 mM) was 60% of that assayed in the presence of $MnCl_2$. This value seems high, as only 10–20% of maximal activity has been observed with $MgCl_2$ alone in other tissues (Hardman *et al.*, 1973), as in this study. The synergistic interaction of $MgCl_2$ or $CaCl_2$ with suboptimal concentrations of $MnCl_2$ (i.e. $[MnCl_2] \leq [GTP]$) has not been previously reported for platelet guanylate cyclase, although similar effects have been observed in many other tissues (Hardman *et al.*, 1973; Garbers *et al.*, 1975; Chrisman *et al.*, 1975). It is probable that the stimulation of guanylate cyclase activity by high concentrations of $MgCl_2$ or $CaCl_2$, when $MnCl_2$ /GTP molar ratios are 1 or less, is due in part to the increase in the concentration of free Mn^{2+} resulting from the formation of an $MgGTP$ or $CaGTP$ complex (Hardman *et al.*, 1973; Garbers *et al.*, 1975). However, the concentration of Mn^{2+} in whole cells is normally in the micromolar range, as compared with millimolar concentrations of Mg^{2+} (Thiers & Vallee, 1957). As the concentration of GTP in the platelet cytosol is about 0.8 mM (calculated from Agarwal & Parks, 1975), it follows that the substrate of platelet guanylate cyclase is likely to be $MgGTP$ and the activating bivalent cation Mg^{2+} . These considerations suggest that the ionic environment in the

platelet may alone limit guanylate cyclase activity to about 15% of the optimum. The possibility remains that low concentrations of free Mn^{2+} may serve as a potentiating cofactor (Garbers *et al.*, 1975).

The marked decrease in the specific activity of guanylate cyclase observed when 50 µl or more of the lysate was assayed was due to inhibition of the enzyme by relatively low-molecular-weight non-protein factors, which could be removed by ultrafiltration of the lysate. Up to about 50% of this inhibitory activity was accounted for by adenine nucleotides, which are well known to inhibit guanylate cyclase (Hardman & Sutherland, 1969; Kimura & Murad, 1974b). Because of the release, during freezing and thawing, of adenine nucleotides from platelet dense bodies, which contain about 60% of the total present in platelets (Holmsen & Day, 1971; Agarwal & Parks, 1975), the concentration of adenine nucleotides present in platelet lysate is likely to be higher than in similar preparations from other tissues. However, in intact platelets the concentration of adenine nucleotides in the metabolic compartment alone (6 mM; Holmsen & Day, 1971) should be sufficient to inhibit guanylate cyclase by more than 95% (Fig. 2). Moreover, our results show that other inhibitory factors are at least as important in platelet lysates and, because of the compartmentation of adenine nucleotides, are likely to be even more important in limiting enzyme activity in intact platelets. These other inhibitory factors have not yet been identified, but, in view of the inhibition of platelet guanylate cyclase by dithiothreitol and glutathione, it is possible that platelet thiols contri-

bute to the inhibitory effect. Thus the combined effects of inhibitory factors and of suboptimal ionic conditions are likely to lower the guanylate cyclase activity in intact platelets to almost negligible values in the absence of activating factors.

Activation of guanylate cyclase in platelet lysates

Preincubation. Incubation of platelet lysate for 1 h at 30°C increased its guanylate cyclase activity by up to about 2-fold (Scheme 1). A time- and temperature-dependent activation of guanylate cyclase has also been observed in human platelet homogenates by Böhme *et al.* (1974) and Glass *et al.* (1977a) and in rat lung supernatants by Chrisman *et al.* (1975) and White *et al.* (1976). Other workers studying this enzyme in human platelets have not reported this phenomenon (Barber, 1976; Rodan & Feinstein, 1976). Thiols, such as dithiothreitol and mercaptoethanol, have been shown to prevent the activation of guanylate cyclase by preincubation (Böhme *et al.*, 1974; Chrisman *et al.*, 1975; White *et al.*, 1976; Glass *et al.*, 1977a), though mercaptoethanol will not reverse activation once it has occurred (White *et al.*, 1976). Our results with dithiothreitol are consistent with both of these findings, but show that in addition this compound markedly inhibited both the basal and preincubation-activated guanylate cyclase activities of platelet lysates. No inhibition of basal activity by thiols was observed in rat lung supernatant, in which the thiols were believed to act

Scheme 1. Relative effects of different combinations of factors that increase platelet guanylate cyclase activity

The approximate activities after the various treatments are given in parentheses as percentages of that of untreated lysate. As indicated in the text, Lubrol PX (1%, w/v) or NaN_3 (10mM) was added to untreated or preincubated lysate 10min before assay. Oleate (1mM) was included in the assay mixtures. The sequence of exposure to activating conditions is shown.

only by preventing a copper- and oxygen-dependent activation process involving the intermediate generation of H_2O_2 (White *et al.*, 1976). Although this mechanism may, in combination with the removal of inhibitory factors, also account for the activation of platelet guanylate cyclase by preincubation, it is apparent from the general inhibitory effects of dithiothreitol and glutathione that thiols may play an additional role in the regulation of the platelet enzyme.

Non-ionic detergents. We have shown that the non-ionic detergents Triton X-100 and Lubrol PX activate platelet guanylate cyclase. There are no previous studies of the effect of Lubrol PX on the platelet enzyme, but Triton X-100 has variously been reported to have an inhibitory effect (Böhme *et al.*, 1974), a slight stimulatory effect (Barber, 1976; Glass *et al.*, 1977a) or no effect at all (Rodan & Feinstein, 1976). Although the reasons for these discrepancies are not clear, in our studies Triton X-100 was substantially less effective than Lubrol PX in stimulating guanylate cyclase activity, particularly at the relatively low concentrations used by earlier workers. Lubrol PX was as effective in increasing the guanylate cyclase activity in 100000g supernatant from lysed platelets as in whole lysate, suggesting that the detergent activates soluble enzyme rather than solubilizes particulate enzyme. Comparable results have been obtained with soluble guanylate cyclase from several mammalian tissues (Hardman *et al.*, 1973; Kimura & Murad, 1974b; Chrisman *et al.*, 1975). Neer & Sukienik (1975) have shown that Lubrol PX activates the soluble form of guanylate cyclase by changing its conformation, possibly as a result of a small amount of detergent binding to specific hydrophobic sites on the enzyme.

Addition of Lubrol PX to a preincubated lysate resulted in a further increase in guanylate cyclase activity of about 2-fold. The activity of this preparation was roughly equivalent to that of a Lubrol PX-treated lysate that had not been preincubated (Scheme 1). In contrast, both Chrisman *et al.* (1975) and White *et al.* (1976) observed that the maximum increases in activity obtained by preincubation or by treatment of rat lung supernatant with Triton X-100 were approximately the same and that the addition of Triton X-100 to a preincubation-activated lysate had no measurable effect on guanylate cyclase activity. The apparent discrepancies between these observations and ours may be due to tissue differences or to the use of a different non-ionic detergent. In either case, our results suggest that the state of activation of guanylate cyclase after preincubation is different from that induced by treatment with Lubrol PX.

Fatty acids. The effects of fatty acids on the activity of platelet guanylate cyclase have attracted interest (Barber, 1976; Glass *et al.*, 1977a; Hidaka & Asano, 1977) because of the specific role in platelet function

of arachidonate and its metabolism to prostaglandin endoperoxides and derivatives (Hamberg & Samuelsson, 1974; Hamberg *et al.*, 1975). However, in our experiments oleate was at least as effective as arachidonate in activating guanylate cyclase in platelet lysates. This finding is in accord with the results reported by Barber (1976), in suggesting that the activation of platelet guanylate cyclase by unsaturated fatty acids is a relatively non-specific effect, perhaps attributable to the surfactant properties of the fatty acids. In contrast, Glass *et al.* (1977a) have reported that platelet guanylate cyclase is specifically activated by arachidonate and closely related polyunsaturated fatty acids through a specific binding site for these fatty acids. Hidaka & Asano (1977) on the other hand believe that the effect of arachidonate depends on its conversion into the hydroperoxy derivative by lipoxygenase. Whether the activation of guanylate cyclase by arachidonate *in vitro* is structurally specific or not, the physiological significance of this effect is doubtful. Thus the induction of platelet aggregation and the release reaction by exogenous arachidonate and the associated increase in the intracellular concentration of cyclic GMP are all blocked by indomethacin, a potent inhibitor of fatty acid cyclo-oxygenase, indicating that in intact platelets all the effects of arachidonate are dependent on its metabolism by the cyclo-oxygenase and not the lipoxygenase pathway (Davies *et al.*, 1976; Glass *et al.*, 1977b). In contrast, activation of guanylate cyclase by arachidonate in platelet lysates was not blocked by indomethacin (the present paper) or aspirin (Glass *et al.*, 1977a). Much higher concentrations of added fatty acids were required to activate guanylate cyclase in our experiments (e.g. 0.1–1 mM) than in those of Barber (1976) or Glass *et al.* (1977a) (e.g. 1–10 μ M). This may be due to the inclusion of albumin in our assays.

The stimulatory effects of oleate and arachidonate were more than additive with the increase in activity caused by preincubation, whereas these fatty acids inhibited the activity of a Lubrol PX-treated lysate (Scheme 1). These experiments suggest that the fatty acids may exert their stimulatory effects on guanylate cyclase by a mechanism that is distinct from those responsible for the effects of preincubation and of Lubrol PX, though in the latter case formation of micelles of mixed composition could have influenced the results. Wallach & Pastan (1976) have reported somewhat different results with membrane-bound guanylate cyclase from fibroblasts. This enzyme was stimulated by a variety of highly amphiphilic fatty acids, including palmitate and oleate, in the presence of Mg^{2+} and of optimal concentrations of Lubrol PX, which were substantially lower than required for optimal activation of the enzyme in platelet lysates. These authors also concluded that fatty acids and Lubrol PX act by different mechanisms.

NaN₃. The activation of guanylate cyclase by *NaN₃* in some but not all tissues was first reported by Kimura *et al.* (1975). Activation of guanylate cyclase by *NaN₃* was time- and temperature-dependent (Kimura *et al.*, 1975) and required a protein activating factor (Mittal *et al.*, 1975), which is or closely resembles catalase (Miki *et al.*, 1976). The stimulatory effects of other compounds, such as sodium nitroprusside and nitric oxide, that do not require a protein activating factor have suggested that *NaN₃* may activate guanylate cyclase by the formation of nitric oxide or nitric oxide-containing compounds (Katsuki *et al.*, 1977; Miki *et al.*, 1977; Arnold *et al.*, 1977). The present study shows that platelet guanylate cyclase can be activated by *NaN₃*, which implies that platelets contain the necessary activating factor. Activation of platelet guanylate cyclase by *NaN₃* resembled that in other tissues in being time- and temperature-dependent. The results suggested that there were two components to this process, one that can be interpreted as due to the slow formation of an active species from *NaN₃* and another that appeared to involve a 'conditioning' of a component of the lysate by preincubation, which enabled *NaN₃* activation of guanylate cyclase to occur more rapidly. As the activity of platelet guanylate cyclase after treatment with *NaN₃* was substantially higher than observed with other activators (Scheme 1), both the mechanism of activation by *NaN₃* and the final state of the enzyme must be distinct.

Subcellular distribution

Determination of the intracellular distribution of guanylate cyclase is crucial to an understanding of the mechanism of activation of this enzyme. The results of Böhme *et al.* (1974), Rodan & Feinstein (1976), Barber (1976) and Glass *et al.* (1977a) indicate that about 90% of the measurable activity of this enzyme is present in the supernatant fraction from disrupted platelets. This distribution pattern was observed whether platelets were disrupted by sonication (Böhme *et al.*, 1974; Rodan & Feinstein, 1976; Glass *et al.*, 1977a), glycerol lysis (Barber, 1976) or freezing and thawing (Böhme *et al.*, 1974). Our results indicate that the guanylate cyclase activity remaining in the particulate fraction was sequestered soluble enzyme that had not been released into the supernatant fraction during lysis. Finally, we were unable to detect latent guanylate cyclase activity on treatment of particulate fractions with Lubrol PX.

The subcellular distribution of guanylate cyclase in mammalian cells has been found to vary from 90% of the total activity in the high-speed supernatant fraction to 90% in the particulate fractions (Hardman & Sutherland, 1969; Goldberg & Haddox, 1977). Investigation of the properties of the enzyme in

these fractions from several tissues has suggested that guanylate cyclase may be present in cells in distinct soluble and particulate forms that could be regulated by independent mechanisms (Kimura & Murad, 1975; Chrisman *et al.*, 1975; Siegel *et al.*, 1976). However, as the platelet enzyme appears to be entirely soluble, the fact that marked increases in platelet cyclic GMP occur in response to aggregating stimuli (Haslam, 1975; Davies *et al.*, 1976; Haslam, 1977) suggests that indirect mechanisms may exist by which extracellular stimuli can activate the soluble enzyme in the cytosol. In support of this view, aggregating agents that increase platelet cyclic GMP concentrations did not, with the exception of arachidonate, increase the guanylate cyclase activity of whole platelet lysate. In this regard, similar results were reported with respect to the effects of aggregating agents on platelet supernatant by Barber (1976).

Conclusions

Our studies, in combination with those of others, indicate that the activity of platelet guanylate cyclase can be modified by a wide variety of factors, most of which appear to act by independent mechanisms. These factors include bivalent cations, adenine nucleotides, unidentified inhibitory factors present in platelets, thiol compounds, an O_2 -dependent activation process (the preincubation effect), non-ionic detergents, unsaturated fatty acids and NaN_3 (or nitric oxide). Several of the variously activated and inhibited states of the enzyme induced by these agents may exist in intact platelets or have a physiological parallel, but at present there is little evidence as to which may be important. Studies of the activation process in intact platelets and of the properties of the activated enzyme within or prepared from intact platelets that have been stimulated by aggregating agents are required to resolve this question.

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