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ARTICLES

A New cGMP Phosphodiesterase Isolated From Bovine Platelets Is Substrate for cAMP- and cGMP-Dependent Protein Kinases: Evidence for a Key Role in the Process of Platelet Activation

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Abstract The biochemical differences among cGMP phosphodiesterases in platelets have not been thoroughly examined, primarily due to the lack of sufficient purified material. This report describes a simple method developed to isolate a specific bovine platelet cGMP phosphodiesterase. This enzyme is cytosolic in its native form and was purified to an apparent homogeneity by ion-exchange chromatography, affinity chromatography, and density gradient centrifugation. Cyclic GMP binds to a "pseudo-site" when the catalytic site is deprived of Mg^{++}. The affinity for cGMP at alkaline pH in presence of EDTA and IBMX (Kd = 60 nM) suggests that the removal of Mg^{++} by EDTA converts the catalytic site to a binding site. A ligand affinity chromatography was designed to take advantage of these features. The core enzyme has a molecular weight 190,000 composed of 2 subunits (MW 95,000) and has a specific activity of 2.5 μmol/min/mg. Moreover, this enzyme was phosphorylated by cAMP- and cGMP-dependent protein kinases, suggesting that its activity could be indirectly regulated by cyclic nucleotides. Agents elevating cGMP and cAMP inhibit platelet activation by inhibiting protein kinase C and thrombin induced hydrolysis of phosphatidylinositol 4,5 diphosphate. The antiaggregating properties of some of these agents might therefore be attributed to the fact that they are inhibitors of phosphodiesterases.

Key words: cGMP, phosphodiesterase, platelets, cAMP, kinase

Platelets contain several distinct phosphodiesterases isoenzymes which cleave preferentially cGMP or cAMP and vary in their substrate affinities [1,2]. Much of the cAMP phosphodiesterase activity in human platelets is due to the cGMP inhibited isoenzyme [3]. However, cGMP phosphodiesterase activities have been only partially characterized [4]. These enzymes in platelets are extremely difficult to purify due to the rare biological material and the proteolytic degradation during the isolation procedure. A major problem has been the absence of method to separate these isoenzymes and to study their functions in the aggregation process. We developed a new approach which uses the presence of a transient high-affinity binding site for cGMP under certain conditions (pH 8.5, presence of EDTA and IBMX) to isolate our enzyme from the others by affinity chromatography. The affinity chromatography was synthetized by coupling the amino group of amino-hexyl Sepharose to the carboxyl group of 2'-o-monosuccinyl 3'5'cGMP. This affinity matrix was used to purify a cGMP phosphodiesterase from bovine platelets corresponding to 7 liters of blood.

On the other hand, an increase in intracellular levels of cAMP and/or cGMP in platelets is associated with the inhibition of change of shape, aggregation, adhesion, and release of granule content [5–7]. The platelet guanylate cyclase can be activated by a variety of agents like nitric oxide or sodium nitroprusside [8–10]. These drugs inhibit platelets' aggregation and release reaction. The physiology of platelets depends on critical concentration of cAMP and cGMP. The importance of phosphodiesterases in platelets is highlighted by the effects of antiaggregating agents like papaverine, dipyridamole, and methyl xanthine—all known as phosphodiesterase

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inhibitors, although their specificity is not clearly determined [12–14].

EXPERIMENTAL PROCEDURES

Materials

(^{8,3}H) guanosine 3′5′cyclic phosphate (24.6 Ci/mmol) was purchased from Amersham, [γ^{32}P] ATP (3000 Ci/mmol) was from Dupont New England Nuclear and [γ^{32}P] cGMP (25 Ci/mmol) from ICN. Crotalus Atrox snake venom, cGMP, cAMP, 3-isobutyl 1 methyl xanthine, theophylline, and dipyrimadole were obtained from Sigma. Zaprinast was a gift from May and Becker (code 22,948). Cilostamine is described in [3] and was synthetized by H. Hidaka, Department of Pharmacology, Mie, University Edsbashi, Japan. Methoxymethyl MIX was a gift from J.N. Wells, Department of Pharmacology, School of Medicine, Vanderbilt University, Nashville. AH Sepharose 4B, N-ethyl N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 2′-o-monosuccinylguanosine 3′5′cyclic monophosphate used for synthetizing the affinity chromatography matrix were purchased from Sigma.

Preparation of Bovine Platelets

Fresh bovine blood from a local slaughterhouse was collected and immediately treated with 0.15 volume of ADC buffer (85 mM trisodium citrate, 111 mM dextrose, 71 mM citric acid) to prevent coagulation. Platelet rich plasma was obtained by centrifugation of the blood in plastic bottles (465 ml) adapted to a Sorvall rotor (type JA-14) at 300*g* for 10 min. This plasma was then centrifuged at 150*g* for 10 min to remove residual contaminant of red cells. Finally, a platelet rich pellet was obtained by centrifugation at 2,500*g* for 10 min. Platelets were removed from any remaining red cells by carefully removing the upper part of the pellet with a rubber policeman, leaving the lower portion containing red cells undisturbed. This pellet equivalent to 7 liters of blood, was resuspended in PEM (sodium phosphate monobasic/dibasic, 10 mM; EDTA, 4 mM; mercaptoethanol (0.2% final) to a total volume of 40 ml, at 4°C. The platelets were disrupted by using a nitrogen bomb decompression after pressurization to 12,500 psi for 20 min. The disrupted platelet suspension was centrifuged at 100,000*g* (38,000 rpm) at 4°C in a SW 41 rotor for 1 hr. The supernatant was diluted 4 times and loaded onto a DEAE Sephacel column (50 ml) equilibrated in PEM. The column was washed with 3 volumes of starting buffer, then eluted with a 140 ml linear gradient of 0 to 250 mM NaCl in PEM at 4°C.

Cyclic GMP Binding Assay

Unless otherwise noted, cGMP binding activity was measured in a 120 μl volume containing 50 μl of PEM plus NaCl from fractions eluted in DEAE column, 50 μl of 1.0 μM [^{3}H] cGMP (1,200,000 cpm), 1 μM cAMP, with or without 0.5 mM 1-methyl 3-isobutylxanthine (IBMX) and 20 μl of sodium phosphate dibasic 0.5 M, pH 8.25. The tubes were incubated at 4°C for 45 min, and the reaction was stopped by the addition of 1 ml of cold saturated ammonium sulfate solution. The tubes were filtered through millipore filters and washed twice with 2 ml of the stop buffer. The filters were dried and counted in a non-aqueous liquid scintillant. Scatchard analysis of the data were performed according to a multistep model.

Determination of Kinetic Parameters

The enzyme concentration was 10 ng/250 μl and the concentration of cGMP varied from 25 nM to 25 μM. The data was graphically represented as V versus V/[cGMP]. (V: micromoles hydrolysed/min/mg of enzyme). Linear curves were obtained, the Y axis intercept giving V_{max} and the value corresponding to $V_{max}/2$ on the X axis intercept yielding Km. The same representation and protocol was used for cAMP as a substrate except that the concentrations of cAMP varied from 1 μM to 100 μM.

Measurement of Phosphodiesterase Activity

Cyclic GMP phosphodiesterase activity was measured according to the method of Wells [15]. The enzyme was added to a final volume of 150 μl containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.33 mg/ml of "metal free" bovine serum albumin, 4.0 μM unlabeled cGMP, and 40 nM [^{3}H] cGMP. After 15 min at 30°C the reaction was stopped by the addition of 20 μl of a mixture containing 10 mM cGMP, 50 mM EDTA, 30 mM theophilline, and 100 mM Tris, pH 7.5. The resulting 5′GMP is converted to guanosine by the addition of 20 μl of 10 mg/ml solution of Crotalus Atrox venom 5′nucleotidase and incubated at 30°C for 10 min. The mixture was chromatographed over a quaternary aminoethyl (QAE) Sephadex and the effluent counted in a liquid scintillation cocktail.

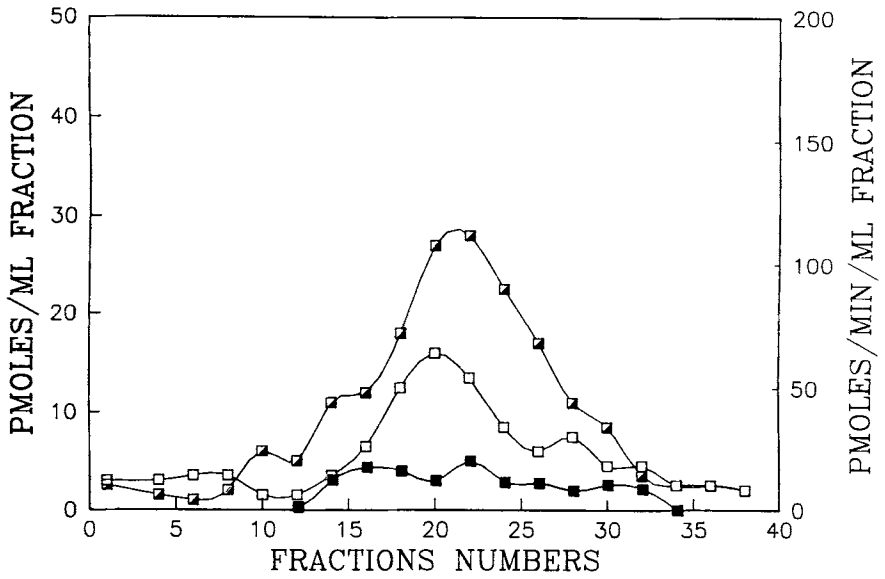


Fig. 1. DEAE Sephacel column chromatography. Disrupted platelets were centrifuged (100,000*g*, 1 hr), and the supernatant was diluted 3 times and applied to DEAE Sephacel column (50 ml), as indicated in Materials and Methods. The elution was developed with a linear gradient of 0 to 250 mM NaCl in PEM (10 mM). Binding assays were performed as described in Experimental Procedures, using 0.5 μM [^{3}H] cGMP and 10 μM cAMP at pH 8.5 (-□-) [^{3}H] cGMP binding in the absence of IBMX (-◪-) [^{3}H] cGMP binding in the presence of 0.5 mM IBMX. The same fractions were assayed for cGMP-dependent kinase activity (-■-).

Synthesis of the Affinity Chromatography Matrix

Five grams of AH Sepharose 4B formed by covalent linkage of 1,6 diaminohexane to Sepharose 4B (Sigma) was swollen for 1 hr and washed on a glass filter with 1 liter of 1 M NaCl solution followed by distilled water. The amount of coupled spacer groups was 10 μmole per ml of swollen gel. One hundred milligrams of 2′-o-monosuccinyl guanosine 3′5′ cyclic monophosphate was dissolved in water, adjusted to pH 4.5, and added to the gel. N-ethyl N′ (3 dimethylaminopropyl) carbodiimide hydrochloride (EDC), corresponding to 10 mg per ml swollen gel, was dissolved in water and mixed gently with gel and ligand for 6 hr. The concentration of the ligand used corresponds approximately to a 1:1 stochiometry of amino group/ligand. Urea-derived and unreacted carbodiimide were extensively washed with 1 M NaCl, and it was determined by UV absorbance at 255 nm that 6.5 μmol of ligand/ml swollen gel were present.

Sucrose Gradient Centrifugation

Linear 5 to 20% sucrose gradient (12 ml) were formed in a buffer containing 10 mM sodium phosphate, 100 mM NaCl, EDTA 2 mM, pH 6.8. Fifty μl of hemoglobin (10 mg/ml) and phosphorylase B (50 mg/ml) were added as internal standards along with 100 μl of the phosphodiesterase sample. After centrifugation in a Beckman SW 41 rotor at 38,000 rpm for 18 hr at 4°C, the gradients were fractionated from the bottom, collecting 0.3 ml fractions with a peristaltic pump. Four centrifuge tubes were thus fractionated and analyzed by gel electrophoresis.

Determination of cGMP Kinase Activity

Cyclic GMP kinase was measured as described previously [16] in presence of 0.5 μM cGMP. Assays were conducted at 30°C for 10 min.

Purification of Bovine Platelet Low Km cGMP Phosphodiesterase

The affinity chromatography of the phosphodiesterase used the cGMP high-affinity binding site generated by alkaline pH, IBMX, and EDTA.

The [^{3}H] cGMP binding activity stimulated by IBMX and alkaline pH was measured on each fraction of DEAE elution. The peak of binding activity eluting at 140 mM NaCl was pooled, diluted 3 times and re-applied to a second DEAE Sephacel column (20 ml) pre-equilibrated with PEM. One hundred milliliters of PEM plus 90 mM NaCl were used to wash the column. The binding activity was then eluted with PEM plus 250 mM NaCl. The pooled samples correspond-

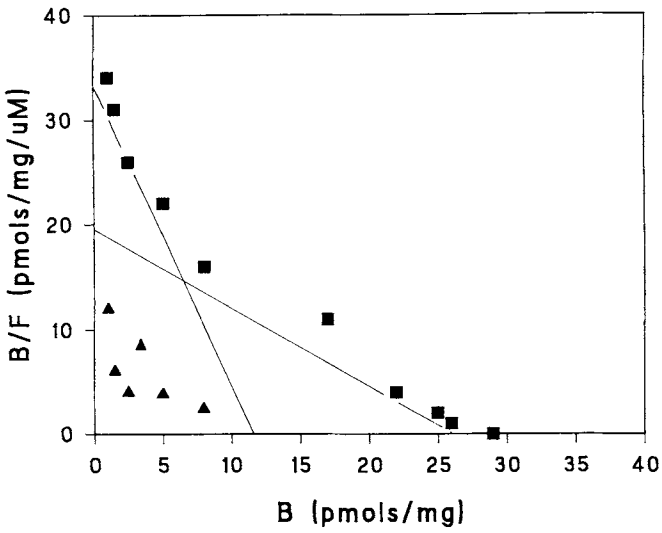


Fig. 2. Scatchard analysis of [^{3}H] cGMP binding to proteins from DEAE fractions performed at pH 7.5. The binding was performed at equilibrium as described in Materials and Methods. After 1 hr at 4°C, 1 ml of cold saturated ammonium sulfate was added and each tube was filtered on a millipore filter, washed, dried, and subjected to scintillation counting. Each point represents duplicate determinations. ■ Represents binding assay with IBMX and ▲ without IBMX.

ing to this binding activity (30 ml) were adjusted to pH 8.5 and IBMX, GMP, adenosine were added to a final concentration 1.1 and 1 mM, respectively. Five milliliters of swollen cGMP-Sepharose were directly added and gently stired with a Pasteur pipette every 10 min for 3 hr at 4°C in order to produce a homogeneous suspension.

Phosphodiesterase Elution From the Affinity Column

The supernatant was discarded after the affinity Sepharose was sedimented. Forty milliliters of PEM plus 500 mM NaCl was used to wash the resin by resuspension with a Pasteur pipette every 30 min. After 2 hr, the supernatant was discarded and fresh solution added. This washing was repeated 3 times for 5 hr at 4°C. The specific elution of the phosphodiesterase was carried out by washing with 5 ml of PEM plus 500 mM NaCl plus cold 10 mM cGMP at 4°C overnight. Because the recovery of the enzyme was poor, another procedure was also used. One milliliter of thiocyanate (1M) was added to the resin for 15 min, then eluted and loaded carefully on top of a sucrose gradient (5 to 20%), and centrifuged 38,000 rpm (100,000*g*) for 18 hr. All steps described above were performed at 4°C.

TABLE I. Inhibition of cGMP Phosphodiesterase Activity by Different Inhibitors*

Inhibitor	IC 50 (μM)
Zaprinast	0.1
Dipyridamole	0.4
IBMX	6
MeOxMeMIX	10
Cilostamide	200
Theophilline	200

*Phosphodiesterase activity was measured as described in Materials and Methods. Results are the average of duplicate determinations.

Due to the dilution of the enzyme in the sucrose gradient, a procedure of concentration was necessary to evaluate its homogeneity. Identical fractions of 0.5 ml from 4 sucrose gradient tubes were pooled and complemented with trichloracetic acid (5% final) and filtered on a millipore filter. The filter was cut around the spot and the proteins were solubilized in 50 μl of 10% SDS.

Determination of Phosphorylation by the C Subunit of cAMP-Dependent Protein Kinase and cGMP-Dependent Protein Kinase

One hundred μl of the peak of phosphodiesterase activity from the sucrose gradient fractionated in 24 tubes (0.3 ml) were incubated with 5 μl of C subunit [diluted in 10-fold PEM

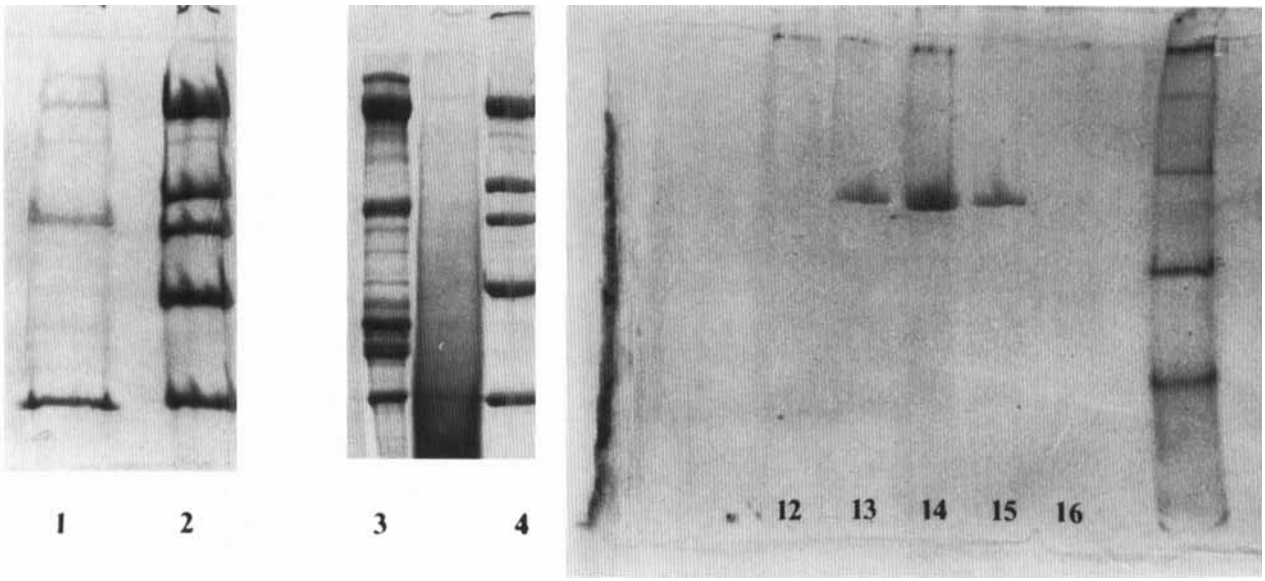


Fig. 3. (**Left**) NaDodSO4 polyacrylamide gel electrophoresis of proteins released from the affinity chromatography. **Lane 1:** Affinity resin was incubated with the pooled fractions of binding activity after two DEAE Sephacel chromatographies, then washed as indicated in Materials and Methods. One hundred μl 10% SDS was added to an aliquot of washed resin and boiled. The supernatant (30 μl) was analyzed by SDS gel electrophoresis on a 8% acrylamide gel (2 mA/gel). **Lane 2** and **Lane 4:** Molecular standards. Myosin (200,000), galactosidase (116,000), phosphorylase B (94,000), Bovine serum albumin (BSA) (66,000), Ovalbumin (42,500). **Lane 3:** Extract of platelets was chromatographed on DEAE Sephacel and fractions corresponding to the binding activity were pooled. The affinity resin was then incubated as indicated above, washed and gel electrophoresis performed for comparison. (Right) NaDoSo4 polyacrylamide gel electrophoresis of purified phosphodiesterase. Sucrose gradients (12 ml) were fractionated in 24 tubes. The corresponding fractions were pooled concentrated, as described in Materials and Methods, and subjected to SDS polyacrylamide gel electrophoresis 8%. The numbers correspond to pooled fractions from 4 sucrose gradients.

immediately, from a concentrated solution of 2.1 mg/ml] with Mg (acetate)^2 (5 mM final) and 1 μl of [γ^{32}P] ATP (0.5 μM final, 100 Ci/mmol) in the presence or absence of cGMP at 0°C for 1 hr. In a separate and similar experiment, 100 μl of each fraction was incubated with 1 μl of cGMP-dependent protein kinase (solution 0.21 mg/ml) with 0.5 μM cGMP. Aliquots were subjected to discontinuous polyacrylamide gel electrophoresis according Laemmli [17] and the amount of [^{32}P] incorporated into the enzyme was determined from autoradiograms of the dried gel.

RESULTS

A cGMP Molecule Linked to a Matrix Via the Hydroxyl Group of the Ribose Conserves the Binding Properties of the Nucleotide to the "pseudo site" of cGMP Phosphodiesterase

In our studies, platelets from freshly collected blood were continuously stirred in citric acid/citrate sodium buffer to keep the pH of the plasma at 6.5 in order to prevent secretion of granular content, aggregation, and coagulation. This cGMP phosphodiesterase was purified from platelets using the high-affinity binding site, generated in presence of EDTA and IBMX at alkaline pH. Figure 1 shows the elution profile of the [^{3}H] cGMP binding activity from the DEAE Sephacel column chromatography. The amount of [^{3}H] cGMP bound to the phosphodiesterase increased twice at pH 8.5 in presence of IBMX. The peak of activity eluted at 140 mM NaCl. Each fraction was also assayed for a cGMP dependent kinase, which can be a potential source of cGMP binding site. As reported elsewhere [4] no substantial activity was found in the different fractions, indicating that in platelets the cGMP-dependent kinase is membrane associated. In order to study this binding in more detail, Scatchard analysis was performed at pH 7.5 in presence of EDTA. We observed that [^{3}H] cGMP binds 2 classes of binding sites. The incubation of [^{3}H] cGMP in the absence of IBMX appears to bind low-affinity binding sites (Kd = 1.5 10^{-6} M). In contrast, higher affinity sites (Kd = 3.5 10^{-6} M). In contrast, higher affinity sites (Kd = 3.5 10^{-7} M) are generated in presence of IBMX. This effect turned out to be

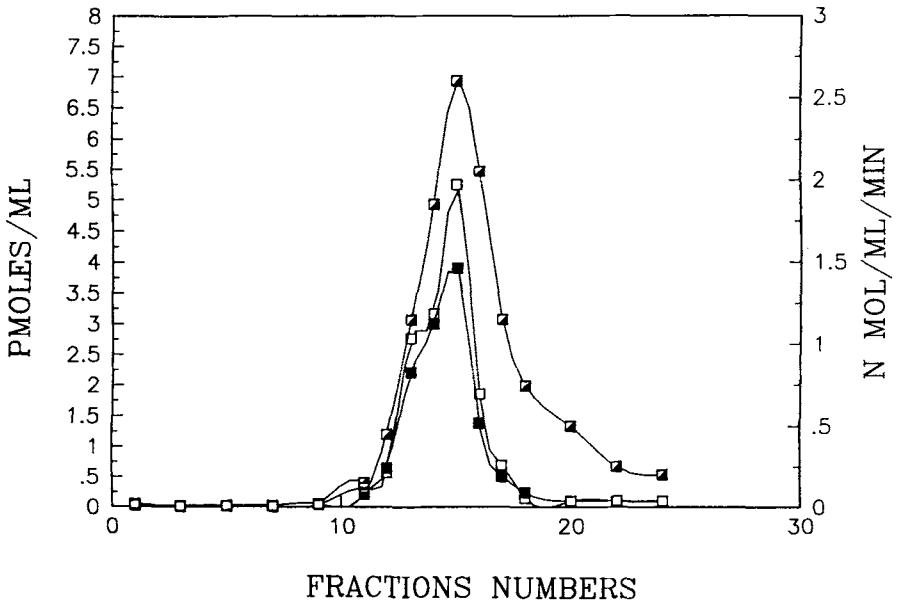


Fig. 4. Cyclic GMP binding and cGMP phosphodiesterase activity profiles obtained with fractions from a sucrose density gradient centrifugation. Cyclic GMP binding was assayed as indicated in Materials and Methods, except [γ^{32}P] cGMP (25 Ci/mmol) was used. [γ^{32}P] cGMP was bound in the absence (-□-) or in the presence (-◪-) of 0.2 mM of IBMX. Each fraction was also assayed for cGMP phosphodiesterase activity (■) as described in Materials and Methods.

specific for this particular phosphodiesterase. The low binding sites could be attributed to other phosphodiesterases, their catalytic sites doubling as binding sites (see Fig. 2).

A second DEAE chromatography was performed with the pooled fractions corresponding to the IBMX stimulated [^{3}H] cGMP binding from the first DEAE elution. The peak of the binding activity was then applied to the 2'o-succinyl cGMP-Sepharose (4 or 5 ml) at pH 8.5 in presence of IBMX and 10 mM cold GMP, 1 mM adenosine. Approximately 80% of the total

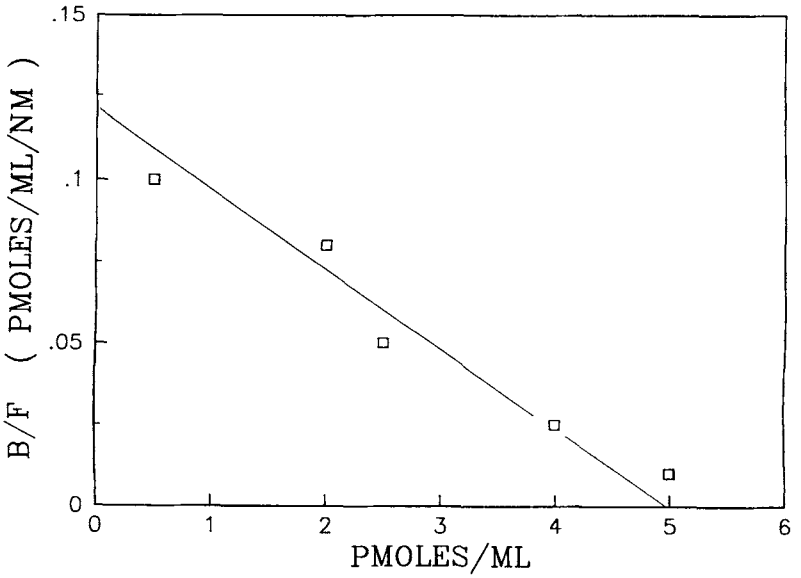


Fig. 5. Scatchard analysis of [γ^{32}P] cGMP binding to purified phosphodiesterase at pH 8.5. The same procedure described in Figure 2 was used, except that the saturation curve was obtained with [γ^{32}P] cGMP (25 Ci/mmol) at pH 8.5.

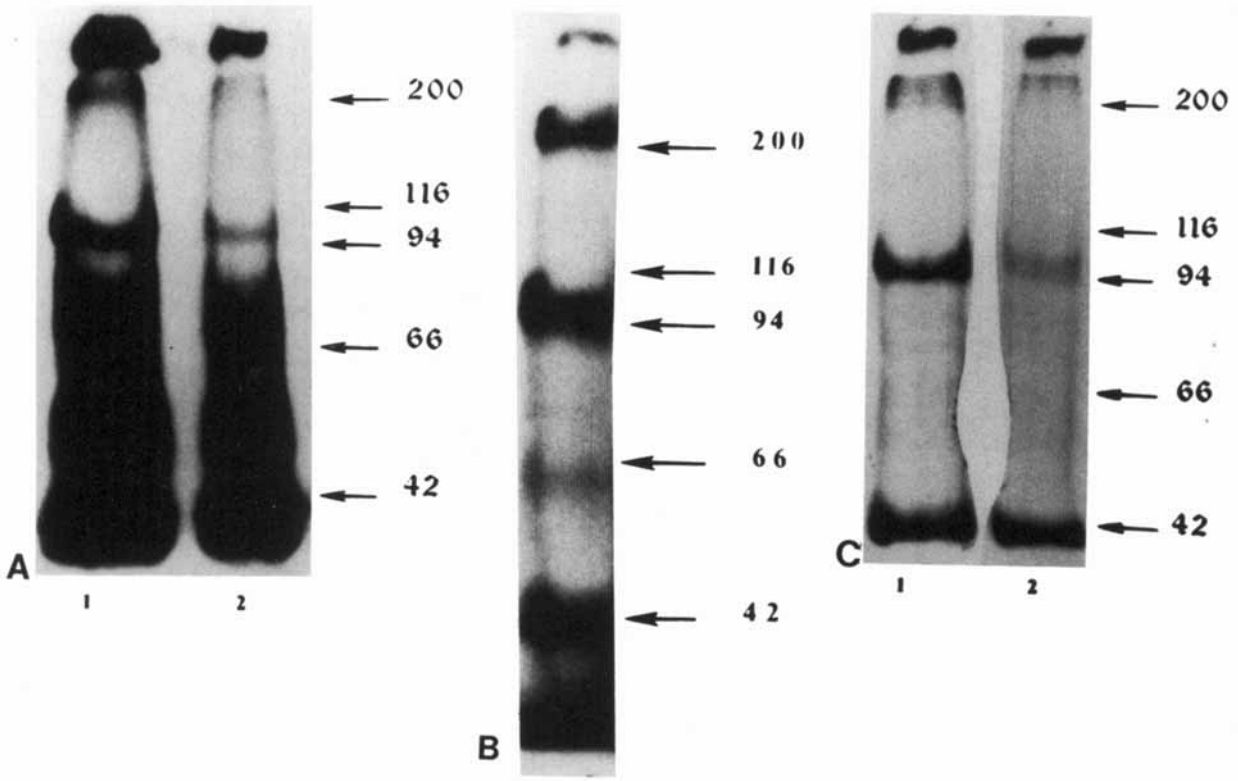


Fig. 6. Phosphorylation of cGMP phosphodiesterase at different steps of purification after two DEAE Sephacel elutions and affinity chromatography. (**A**) The affinity resin incubated with extract of platelets and washed, was treated with a chaotropic agent (2 ml of 1 M thiocyanate). After 10 min, the eluate (500 μl) was loaded on a G 25 Sephadex column (12 ml) equilibrated with PEM buffer. The phosphodiesterase activity in the void volume was incubated in presence of the C subunit (0.005 mg/ml) and 0.5 μM [γ^{32}P] ATP (100 Ci/mmol) with cGMP (10 μM) (**1**) or without cGMP (**2**). The proteins were concentrated by filtration on a millipore filter, and 100 μl 10% SDS was added to the excised spot. (**B**) The affinity resin was bound to the enzyme and then incubated in PEM 500 mM NaCl plus 10 mM cold cGMP overnight at 4°C. The supernatant (1 ml) was loaded on the top of G25 column (15 ml). The pooled fractions corresponding to the phosphodiesterase activity (1.5 ml) were incubated with the C subunit (0.005 mg/ml) and 0.5 μM [γ^{32}P] ATP (100 Ci/mmol). The sample of proteins were concentrated by filtration on a millipore filter and 100 μl of 10% SDS was added to the excised spot. Forty μl of this solution was subjected to electrophoresis analysis. (**C**) The affinity resin, incubated with fractions eluted from the DEAE Sephacel chromatography, was washed and treated with a chaotropic agent (2 ml of 1 M Na thiocyanate). After 10 min the eluate (1 ml) was loaded on the top of 4 sucrose gradients (12 ml) and run for 18 hr at 38,000 rpm. The gradients were fractionated and the fractions corresponding to the peak of activity was phosphorylated by the C subunit in the presence (**1**) or in the absence (**2**) of cGMP (10 μM).

binding activity was immobilized on the matrix (Table 1). The release of protein was carried out in two different ways. The first approach was an overnight incubation with a large excess of cold cGMP. The supernatant was processed through a G25 column to remove the excess of cGMP. A phosphodiesterase activity-like was found and measured in the void volume (data not shown). These fractions were concentrated and gel electrophoresis was performed. Five bands were found (data not shown). Because the identical bands were obtained when an aliquot of the same resin was boiled in presence of 10% SDS or when proteins were released by chaotropic salt (1 M Na thiocyanate) and because the specific release by incubation with cold cGMP was very low, a second protocol was utilized. Thus, three major bands, released by chaotropic salts, were observed when a second DEAE chromatography was performed before the affinity chromatography, (Fig. 3, left). To complete the purification to apparent homogeneity, this salt released material was loaded on a sucrose gradient and ultracentrifuged. cGMP-binding and cGMP phosphodiesterase activity was measured corresponding to a molecular weight just higher to phosphorylase B (the molecular weight was calculated at Mr 190,000). Small molecules, as well as the salts, remained at the top the gradient, performing separation and renaturation of the

enzyme. We observed that binding and phosphodiesterase activities coincided (Fig. 4).

The Low Km of This cGMP Phosphodiesterase Suggests That the Binding Site Is a Catalytic Site Reversibly Blocked

The band of the sucrose gradient corresponding to phosphodiesterase activity was used for further determinations after complementation with Mg^{++}. The specific activity was found to be 2.5 μmol/min/mg when the substrate is at 5 μM and the V_{max} was found to 2.9 μmol/min/mg when the substrate is at 50 μM at 30°C. The Km was found to be 140 nM at 4°C and 220 nM at 30°C. The equilibrium for the binding assay was achieved after 15 min at 4°C and the IC 50 of the dissociation was reached after 2 hr. A Scatchard analysis, using [y^{32}P] cGMP under optimal conditions, shows a unique binding site with an affinity Kd of 60 nM (Fig. 5). We observed that the molecular weight is 95,000 in electrophoresis analysis, which implies that the enzyme is an homodimer. In presence of Mg^{++}, neutral pH, without IBMX, this binding activity was absent. It is remarkable that the enzymatic activity survived transient treatment with 1 M thiocyanate and recovered, at least partially, by renaturation in sucrose gradient (Fig 3, right).

The Phosphorylation by the cGMP- and cAMP-Dependent Protein Kinases Suggests That Cyclic Nucleotides Indirectly Regulate This Low Km Phosphodiesterase

This enzyme was phosphorylated with C subunits of cAMP- and cGMP-dependent kinases. The results are shown in Figures 6 and 7. Both enzymes are potential candidates for "in vivo" phosphorylation. We noted that the presence of cGMP was a pre-requisite for a rapid phosphorylation by the C subunit. In Figure 6, the low band corresponds with autophosphorylation of the C subunit. The high band (200,000 kD) could be related to the native form not completely separated in monomer forms. Phosphorylation experiments performed before the sucrose gradient step, did not change the migration of this enzyme suggesting that the dimeric form was not modified. In Figure 7, the 70 kD band corresponds with the autophosphorylation of cGMP protein kinase. The specific activity of this phosphodiesterase, as well as the specificity of cleavage cGMP/cAMP, was unchanged when phosphorylation was performed (data not shown).

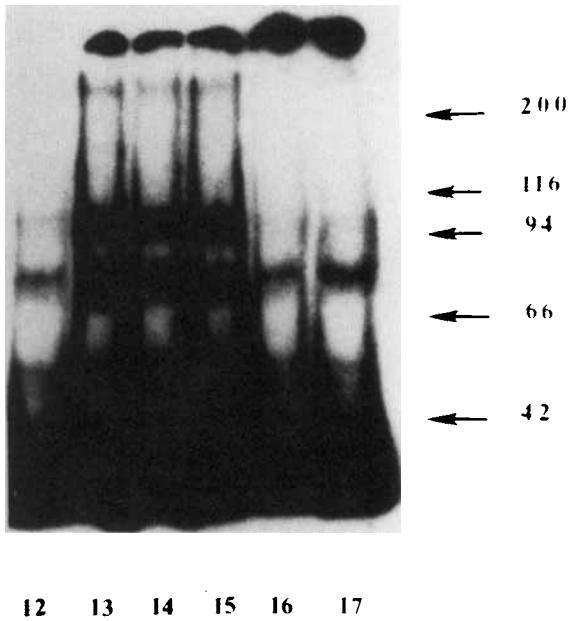


Fig. 7. In vitro phosphorylation of the phosphodiesterase by cGMP-dependent protein kinase. Incorporation of [y^{32}P] phosphate into the band MW: 95,000 from fractions of sucrose gradient. The numbers (13–15) correspond to fractions where phosphodiesterase activity was detected. Proteins were phosphorylated by cGMP-dependent protein kinase (0.005 mg/ml) in the presence of 10 μM cGMP and 0.5 μM [y^{32}P] ATP (100 Ci/mmol). The MW 70,000 corresponds to the autophosphorylated cGMP-dependent protein kinase.

Antiaggregating Drugs Like Zaprinast and Dipyramidole Are Inhibitors of the Low Km cGMP Phosphodiesterase

Different drugs inhibited basal activity of this phosphodiesterase. Maximal inhibition occurred with Zaprinast and Dipyramidole (IC^{50} = 0.1 and 0.4 μM, respectively). IBMX and MeOxMe MIX were inhibitory only at higher concentration (IC^{50} = 6 and 10 μM, respectively). In the presence of theophilline and cilostamine the phosphodiesterase activity was unaffected up to 100 μM (Table I). We have also investigated the effect of pH on binding and phosphodiesterase activity. The hydrolysis activity was maximum around pH 8. The optimal conditions for binding occurred at pH 8.5 (Table II). The specificity of this phosphodiesterase for cGMP was tested. The cGMP hydrolysis rate was unchanged in presence of 40-fold excess of cAMP (data not shown). We found also that 8-bromo-cGMP (1 μM) did not compete for the binding site in our standard conditions. This suggest that the steric hindrance related to the puric part of cGMP determines the strict specificity for catalytic activity and subsequent binding activity.

TABLE II. Effects of pH on Binding and Catalytic Properties of the Purified Enzyme*

pH	Km	Catalytic activity (%)	Binding activity (%)
6	—	33	24
6.5	—	58	28
7	—	82	43
7.5	0.14	90	45
8	—	100	88
8.5	—	82	100
9	—	72	68
9.5	—	53	54

*The results are expressed as a percentage of the maximum value and represent the average of duplicate experiments. These experiments were carried out at 4°C, which corresponds to standard conditions of binding.

DISCUSSION

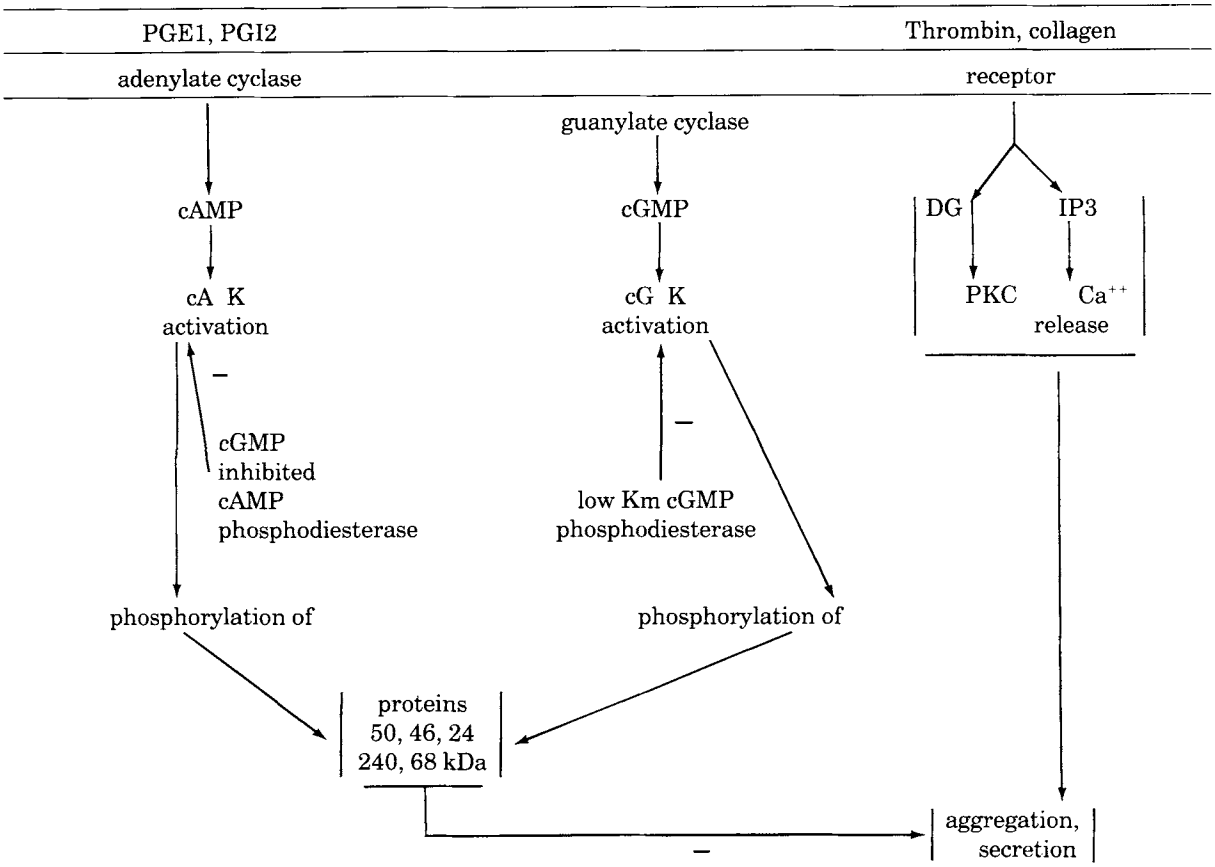
Table III summarizes the purification of this phosphodiesterase from 7 liters of tissue. The procedure depended on two successive DEAE Sephacel chromatographic steps, an affinity chromatographic step and an ultracentrifugation on sucrose gradient. The results therefore suggested that this enzyme is dimeric and is composed of by two homologous monomers. Platelets contain several phosphodiesterase activities which have been described [18–21]. A cGMP inhibited cAMP phosphodiesterase appears to be the essential enzyme to mediate a negative feedback mechanism to reverse aggregatory signals [22]. In contrast, the regulation of cGMP levels are largely unknown. Usual concentrations of cAMP and cGMP are 25 pmol/10^{-9} platelets and 2.5 pmol/10^{-9} platelets, which correspond to approximately 4.4 μM and 0.44 μM, respectively [23]. Prostacyclin, a potent activator of adenylate cyclase, is known to inhibit thrombin induced formation of inositol triphosphate by accumulating cAMP [24]. Drugs that increased cGMP inactivate cGMP-inhibited cAMP phosphodiesterases (characterized as a low Km enzyme and major isoenzyme in platelets) leading to elevated cAMP levels and consecutive activation of cA kinase [25]. Therefore, cGMP regulates the level of cAMP through the cGMP inhibited cAMP phosphodiesterase. Our enzyme works in a range of low concentration of cGMP which is consistent with the level of unactivated and unstimulated platelets (0.4 μM). This enzyme is highly specific for this substrate and indirectly regulates the cGMP-inhibited cAMP phosphodiesterase which works in low concentrations of cAMP. These interactions are summarized in Table 4. Therefore, this enzyme is a candidate for a key role in the process of aggregation by indirectly blocking the inhibitory effects of both cAMP- and cGMP-dependent protein kinases. The consequence of the phosphorylation of this enzyme by these kinases is still unclear. We can postulate a regulation by a negative feed back increasing the catalytic efficiency when concentration of cGMP or cAMP increases. The regulation of this enzyme might be directly mediated by cAMP- or cGMP-dependent kinase. This assumption is supported by the "in vitro" phosphorylation data. This enzyme was also inhibited by zaprinast and dipyramidole. These drugs can induce inhibition of aggregation [4]. On the other hand, we note extensive homology between our enzyme and another isolated cGMP-binding protein phosphodiesterase from bovine lung [26–28]. The profile of inhibitors, the presence of a high-

TABLE III. Summary of the Purification of the Enzyme*

	Units binding (%)	Specific activity (binding)	Specific activity (catalytic)
DEAE (1)	100	19.5 pmol/mg	1.88 10^{-3}
DEAE (2)	71	68.2	12.8 10^{-3}
Affinity chromatography (quantity not immobilized)	22		
Sucrose gradient	8.8	5 nmoles/mg	2.5 μmol/min/mg

*The table represents a single preparation but is representative of other similar preparations. The % recovery after elution of affinity chromatography was not calculated, since the protein was released with a chaotropic agent for 10 min, which transiently abolishes the catalytic activity and increases the affinity of cGMP binding. DEAE (1): The determinations were performed with fractions 18–25. These pooled fractions correspond to 11.5% of the total proteins of the platelet extract. DEAE (2): A second DEAE chromatography was performed with the fractions 18–25 of the first one. The column was washed with 90 mM NaCl before elution. The yield of purification was about 2,000 for the binding activity and 13,000 for the catalytic activity.

TABLE IV. Model Summarizing the Regulatory Role of cAMP and cGMP in Platelet Function



PGE1, PGI2 prostaglandin E1 and I2; DG 1,2 diacylglycerol; IP3 inositol triphosphate; PKC protein kinase C, negative regulation.

TABLE V. Characterization of the Solubilized Low Km cGMPPhosphodiesterase in Platelets

Substrate specificity	Hydrolyses cyclic GMP specifically
Km for cyclic GMP	0.22
Km for cyclic AMP	40
Vmax for cGMP	2.9 μmol/min/mg
Vmax for cAMP	1.3 μmol/min/mg
Stimulation by cAMP	No
Activation by calmodulin and Ca^{++}	No
Kinetics	Linear
Mg^{++} dependent	Yes
Homodimer	190,000 kD
High affinity binding to cGMP	Require EDTA, IBMX, and alkaline pH (not found in presence of Mg^{++} at neutral pH)

affinity binding site in elevated pH, and molecular weight are analoguous. Although the Km is different (.22 μM versus 5 μM) and the presence of a binding site dissociated of a catalytic site not substantiated in our case, our studies suggest that both enzymes belong to a close family of cGMP phosphodiesterases. The features of this enzyme are summarized in Table V. Chaotropic agents were found to increase the binding. This property was used to washed the resin with 500 mM NaCl while preventing the enzyme from being released. The second opportunity was the apparent stability of this enzyme after transient passage in Na thiocyanate salt (1 M). The interesting property was the phosphorylation induced by C subunit of cA kinase occured only in presence of cGMP. This element was a useful test to follow the purification. We can postulate that the occupancy of the catalytic site directs the phosphorylation.

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