

cAMP-mediated phosphorylation of the low- K_m cAMP phosphodiesterase markedly stimulates its catalytic activity

(cyclic nucleotides/cAMP-dependent protein kinase/forskolin/immunoblots)

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ABSTRACT Treatment of intact human platelets with the adenylate cyclase agonist forskolin (100 μ M) resulted in an increase in cAMP phosphodiesterase activity in freeze-thaw lysates. When the low- K_m (high affinity), cGMP-inhibited cAMP phosphodiesterase was isolated from such lysates by blue dextran-Sepharose chromatography, the specific activity of the enzyme was increased an average of 11-fold over similarly processed control platelets. The increase in the low- K_m , cGMP-inhibited cAMP phosphodiesterase activity was inhibited when platelets were incubated with the protein kinase inhibitor H-8 prior to treatment with forskolin, suggesting that the stimulation of cAMP phosphodiesterase activity involved a cAMP-dependent phosphorylation. When intact platelets that had been prelabeled with ³²P_i were treated with forskolin and the low- K_m , cGMP-inhibited phosphodiesterase was isolated by blue dextran-Sepharose chromatography, a protein of 110,000 kDa was phosphorylated. By using a monospecific antiserum to the purified phosphodiesterase, this protein was shown to be the low- K_m , cGMP-inhibited cAMP phosphodiesterase by electrophoretic transfer blot (Western blot) analysis and by immunoprecipitation. The stable prostacyclin analog iloprost also stimulated the low- K_m cAMP phosphodiesterase activity about 2-fold and caused phosphorylation of the enzyme. These results suggest that phosphorylation of the low- K_m , cGMP-inhibited phosphodiesterase may be an important regulatory mechanism for this enzyme in platelets.

An increased intracellular level of cAMP is associated with inhibition of platelet responses to agonists, including such responses as shape change, aggregation, adhesion, and granule release (1-3). Thus the regulation of the enzymes involved in cAMP formation and degradation strongly influences the participation of platelets in physiological hemostasis and pathological thrombosis. The control of adenylate cyclase which catalyzes the conversion of ATP to cAMP has been studied in considerable detail (4). Much less is known about the regulation of cAMP catabolism.

The hydrolysis of the intracellular messengers cAMP and cGMP is carried out by several different cyclic nucleotide phosphodiesterases. These enzymes differ in their substrate specificities, kinetic characteristics, physical properties, and regulation (5-7). Most cells contain several of these forms of cyclic nucleotide phosphodiesterase but the role of each of these multiple species of phosphodiesterase in cells is still unclear.

Human platelets have been reported to contain three different forms of cyclic nucleotide phosphodiesterase activity (8). The first is relatively specific for cGMP, the second is a cGMP-stimulated phosphodiesterase that hydrolyzes

cAMP and cGMP equally well, and the third displays a low K_m (high affinity) for cAMP but hydrolyzes cGMP poorly. We have previously reported a purification of this third form of phosphodiesterase from human platelets (9). The purified enzyme had a low K_m (high affinity) for cAMP and was inhibited competitively by cGMP. Kinetically similar forms of phosphodiesterase activity have been described in a number of cell types (10-13).

Several mechanisms exist for the control of the activities of the various forms of phosphodiesterase in cells. Certain cyclic nucleotide phosphodiesterases are sensitive to activation by Ca²⁺/calmodulin, some are sensitive to stimulation by cGMP, and others appear to be hormonally modulated (5-7). In this paper we report that incubation of intact human platelets with the plant diterpene forskolin or iloprost (ZK 36374), a stable analog of the naturally occurring potent prostaglandin I₂ (prostacyclin), results in stimulation of the activity of the low- K_m (high affinity) cAMP phosphodiesterase and phosphorylation of the phosphodiesterase molecule. Since both agents lead to a rise in platelet cAMP by stimulating adenylate cyclase by different mechanisms, these results provide at least a partial explanation for the apparent desensitization after exposure to these agonists. A preliminary account of this work has been presented (14).

MATERIALS AND METHODS

Determination of cAMP Phosphodiesterase Activity. Phosphodiesterase activity was determined as described using the Dowex column procedure described by Thompson *et al.* (15) or the BaSO₄ procedure of Sinha *et al.* (16). All assays were done at 1 μ M cAMP unless noted.

Preparation of Antiserum. Low- K_m cAMP phosphodiesterase was purified from outdated platelet concentrates as described (9). After an initial bleeding to obtain preimmune serum, a young nulliparous female rabbit was inoculated with 100-150 μ g of the purified phosphodiesterase in complete Freund's adjuvant using multiple intradermal injections as described by Vaitukaitis (17). After 10 weeks the rabbit was given booster injections with \approx 50 μ g of low- K_m cAMP phosphodiesterase in complete Freund's adjuvant. Two weeks after reimmunization blood was drawn from the central ear artery, placed in glass tubes without anticoagulants, incubated at 37°C for 1.5 hr, and then stored overnight at 4°C. The serum removed from the clot was heated at 56°C for 0.5 hr and stored at -70°C.

Neutralization of cAMP Phosphodiesterase Activity by Antiserum. The purified phosphodiesterase was diluted in 125 mM Tris-HCl (pH 7.5) containing 20 mM MgCl₂ and 1 mg of bovine serum albumin per ml. Appropriate dilutions of the

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antiserum were added to the enzyme, incubated for 3.5 hr at 25°C, and assayed for cAMP phosphodiesterase activity.

ELISA of Antiserum with Purified cAMP Phosphodiesterases. ELISA measurements were performed as described by Engvall and Perlmann (18). ELISA cuvettes (Gilford) were coated with purified phosphodiesterases, blocked with 1% bovine serum albumin, and incubated with a 1:500 dilution of the antiserum. After washing the cuvettes, immune complex formation was detected with a goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) using *p*-nitrophenyl phosphate as the substrate.

Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting. NaDodSO₄-containing polyacrylamide gels were run as described by Laemmli (19). Gels were blotted to nitrocellulose as described by Towbin *et al.* (20), blocked with bovine serum albumin or casein, incubated with a 1:500 dilution of antiserum overnight at 4°C, washed, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad). The bands were then visualized by reaction of 4-chloro-1-naphthol and H₂O₂ (21).

Forskolin and Iloprost Treatment of Platelets and Isolation of Low-K_m cAMP Phosphodiesterase. Platelet-rich plasma was prepared from freshly drawn blood anticoagulated with acid citrate dextrose and separated from red blood cells by centrifugation at 120 × *g* for 30 min. The pH of the plasma was adjusted to 6.5 with citric acid and the platelets were pelleted by centrifugation at 1100 × *g* for 20 min. The platelet pellet was resuspended at a concentration of 10¹⁰ platelets per ml in 50 mM Tris·HCl (pH 7.5) containing 20 mM MgCl₂, 0.15 M NaCl, 10 mM benzamide, 2 mM EGTA, 5 mM ϵ -aminocaproic acid, 20 μ M 7-amino-1-chloro-3-tosylamido-2-heptanone, 5 μ M pepstatin A, and 40 μ M leupeptin (buffer A). Forskolin (10 mM in 95% ethanol) or iloprost (in water) was added to 1-ml samples (10¹⁰ platelets) to give a final concentration of 50 or 100 μ M forskolin or 0.1 μ M iloprost. The sample was incubated at 30°C for 5–10 min with forskolin or for 4 min with iloprost. An equal volume of buffer A without MgCl₂ but containing 10 mM NaF and 20 mM sodium pyrophosphate was then added and the sample was mixed briefly and frozen in a dry ice bath. The sample was thawed and centrifuged for 2 min at 12,000 × *g* at 4°C and the supernatant was applied to a 1-ml blue dextran-Sepharose column previously equilibrated with buffer A. After the sample was added the column was washed with at least 5 ml of buffer A and then the phosphodiesterase was eluted with 0.3 mM cAMP in buffer A. The column fractions were assayed for cAMP phosphodiesterase activity at 1 μ M cAMP as described (9) and fractions containing phosphodiesterase activity were pooled and stored at 0–4°C.

Samples to be analyzed on PAGE were lyophilized and then resuspended in the sample buffer for NaDodSO₄ gels (19), heated at 100°C for 2–3 min, cooled, and applied to the gel.

³²P Labeling of Platelets. The platelet pellet was resuspended in 10 mM Hepes (pH 6.6) containing 1 mM MgCl₂, 0.145 M NaCl, 5 mM KCl, 20 mM glucose, 0.5 unit of hirudin per ml, and 10 μ M D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone at a concentration of 5 × 10⁹ platelets per ml. Carrier-free ³²PO₄ was added at 0.25 mCi/ml (1 Ci = 37 GBq) and the suspension was incubated at 37°C for 1 hr with occasional mixing. The platelet suspension was centrifuged at 1100 × *g* for 20 min, and the platelet pellet was resuspended in buffer A at 10¹⁰ platelets per ml and incubated with forskolin or iloprost as described above.

Gels and nitrocellulose blots of ³²P-labeled samples were analyzed for ³²P labeling by autoradiography.

Immunoisolation of Low-K_m cAMP Phosphodiesterase from Platelet Lysate. Immunoprecipitation using anti-low-K_m cAMP phosphodiesterase antiserum was performed by using heat-inactivated, formalin-fixed *Staphylococcus aureus* (Cowen I) cells (Pansorbin, Calbiochem) as described by Kessler (22).

Platelets preincubated with ³²PO₄ were treated with forskolin and lysed as described above, and cellular debris was removed by centrifugation (12,000 × *g*, 10 min). One-fifth volume of a 10% suspension of bovine serum albumin-washed *S. aureus* cells was added to the lysate and incubated for 2 hr at 4°C with gentle mixing. This preincubation was done to reduce nonspecific binding to the *S. aureus* cells. After removal of the *S. aureus* cells, antiserum was added to the lysate (1:50 dilution) and incubated overnight at 4°C. One-fourth volume of a 10% suspension of bovine serum albumin-washed *S. aureus* cells was then added to the lysate and incubated for 4 hr at 4°C. The cells were isolated by centrifugation, washed, resuspended in the sample buffer for NaDodSO₄ gels (19), and prepared for electrophoresis.

RESULTS AND DISCUSSION

Characteristics of a Monospecific Polyclonal Antiserum to Low-K_m (High Affinity) cAMP Phosphodiesterase. Our approach was to demonstrate that phosphorylation occurred in intact platelets as a response to elevated intracellular levels of cAMP. To achieve this we first prepared a monospecific polyclonal antiserum against the low-K_m cAMP phosphodiesterase from human platelets in rabbits. When the antiserum was employed in an immunoblot analysis of a whole platelet lysate, bands were observed at 110 kDa and 75 kDa (Fig. 1, lane 1). When the immunoblot of the lysate was probed with the corresponding preimmune serum the band at 75 kDa was stained (Fig. 1, lane 2). Thus an antibody to this unknown protein was present in the rabbit prior to immunization and is thus irrelevant. The band seen at 110 kDa is the expected monomer molecular mass of the low-K_m cAMP phosphodiesterase in agreement with the results of Beavo and co-workers (11, 23).

When the platelet lysate was passed over a blue dextran-Sepharose column and the phosphodiesterase was eluted with cAMP (see *Materials and Methods*), only a single band at 110 kDa was observed on an immunoblot of the proteins separated by NaDodSO₄/PAGE (Fig. 1, lane 3). The 75-kDa protein did not bind to the blue dextran-Sepharose column and was recovered in the wash-through fraction (data not shown).

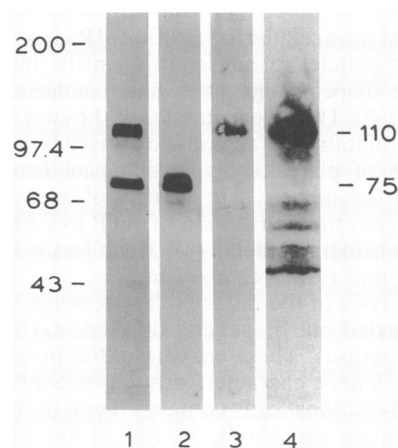


FIG. 1. Immunoblot analysis of human platelet lysate. Samples of a whole human platelet lysate (lanes 1 and 2, from 5 × 10⁹ platelets per lane) or of ³²P-labeled, blue dextran-Sepharose-purified low-K_m cAMP phosphodiesterase (lane 3, from 10¹⁰ platelets) were run on a 7.5% NaDodSO₄-containing polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose (20). The blot was probed with the anti-low-K_m cAMP phosphodiesterase antiserum (lanes 1 and 3) or with the corresponding preimmune serum (lane 2). Lane 4 is an autoradiogram of ³²P labeling of the blot shown in lane 3. Molecular masses are given in kDa.

Further evidence that the antiserum recognized the low- K_m cAMP phosphodiesterase was derived from experiments indicating that the antiserum neutralized the enzymatic activity >90%, whereas the preimmune serum did not inhibit the enzyme (data not shown). The binding of the antiserum was proportional to the concentration of the antigen, purified low- K_m cAMP phosphodiesterase, as detected in an ELISA (Fig. 2). In contrast, no reaction was observed when a purified cGMP-stimulated phosphodiesterase from human platelets (24) was used as the primary antigen (Fig. 2). These results indicate that these two cAMP phosphodiesterase activities found in human platelets are immunologically distinct proteins.

The immunoblot results indicate that the monomer molecular mass of the low- K_m cAMP phosphodiesterase from human platelets is 110 kDa. However, we have previously reported a monomer molecular mass of 61 kDa for the low- K_m cAMP phosphodiesterase purified from platelets derived from outdated platelet concentrates (9). Subsequently, Harrison *et al.* (11) reported the purification of a cAMP phosphodiesterase from bovine cardiac muscle with properties similar to the platelet enzyme but with a monomer molecular mass of 110 kDa. When antibodies raised against this bovine phosphodiesterase were used to probe immunoblots of human and bovine platelet lysates, single reactive bands at 100 kDa were observed in both lysates (23). However, when the bovine platelet lysate was incubated at room temperature for 3–6 hr prior to gel analysis, the phosphodiesterase appeared to undergo sequential proteolysis to 80-kDa and then 60-kDa peptides (23). The evidence from the studies of the bovine cardiac enzyme as well as the results described above with the antiserum against the purified human platelet phosphodiesterase indicate that the 61-kDa form of the low- K_m cAMP phosphodiesterase that we purified from outdated platelet concentrates was a proteolytic fragment of the enzyme. When the antiserum against the 61-kDa form was used to probe an immunoblot of a lysate prepared from platelets isolated from outdated platelet concentrates, the major band was at 110 kDa with a weakly staining band at 60 kDa (data not shown), suggesting that most of the proteolysis occurred during the course of the purification in spite of the presence of a mixture of protease inhibitors in all buffers (9).

Stimulation of Low- K_m cAMP Phosphodiesterase by Forskolin. Treatment of intact rat and human platelets with prostaglandin E_1 or prostacyclin has been reported to stim-

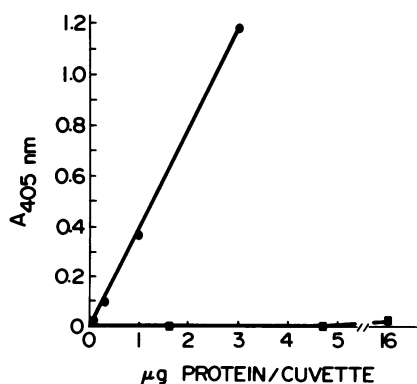


FIG. 2. ELISA of low- K_m cAMP phosphodiesterase and cGMP-stimulated phosphodiesterase from human platelets. Reaction of the anti-low- K_m cAMP phosphodiesterase antiserum with the phosphodiesterases was detected by using an alkaline phosphatase-conjugated secondary antibody. The reaction was terminated after a 10-min incubation with *p*-nitrophenyl phosphate and the absorbance was measured at 405 nm. The results are the average of triplicate determinations. ●, Low- K_m cAMP phosphodiesterase; ■, cGMP-stimulated phosphodiesterase.

ulate cAMP phosphodiesterase activity in platelet lysates (25–27). Treatment of rat platelets with the plant diterpene forskolin, which directly stimulates the catalytic subunit of adenylate cyclase, increased cAMP and cGMP phosphodiesterase activities (28). The enhanced activity was maintained through partial purification of these enzymes, suggesting that the stimulation was due to covalent modification of the enzyme (26–28). Although these authors postulated that this stimulation was due to an intermediate phosphorylation by a cAMP-dependent protein kinase (26–28), no direct evidence of phosphorylation of the phosphodiesterases was demonstrated.

To test this hypothesis we incubated intact human platelets with 50–100 μ M forskolin for 5–10 min at 30°C and disrupted the cells by freezing and thawing. This concentration of forskolin is known to elevate cAMP about 30-fold by direct stimulation of the catalytic subunit of adenylate cyclase (29). Treatment of human platelets with forskolin resulted in a 3- to 4-fold increase in cAMP phosphodiesterase activity in the platelet lysates measured with 1 μ M cAMP. To determine which of the two forms of cAMP phosphodiesterase activity present in human platelets (9) was affected, we separated the phosphodiesterases by blue dextran-Sepharose chromatography. Under the conditions used, the low- K_m cAMP phosphodiesterase bound to the blue dextran column and was eluted with cAMP. In contrast, the cGMP-stimulated phosphodiesterase passed through the column without binding, allowing a rapid separation of these two cAMP phosphodiesterase activities. When the phosphodiesterases were separated, the specific activity of the low- K_m cAMP phosphodiesterase from the forskolin-treated cells was stimulated 11.3-fold (\pm 3.8-fold) over that from control platelets. The degree of stimulation varied with platelet preparations from about 3- to 13-fold. Addition of forskolin directly to platelet lysates and to assays of the blue dextran-Sepharose-purified enzyme resulted in no change in cAMP phosphodiesterase activity (data not shown).

Involvement of cAMP-Dependent Protein Kinase in the Stimulation of Low- K_m cAMP Phosphodiesterase. To determine if the stimulation of the low- K_m cAMP phosphodiesterase activity required the activation of a protein kinase, intact platelets were preincubated for 10 min at 30°C with the protein kinase inhibitor H-8 (100 μ M) prior to treatment of the platelets with forskolin. H-8 has been reported to inhibit cAMP-dependent protein kinase (30). In the experiment depicted in Fig. 3, the increase in the blue dextran-purified low- K_m cAMP phosphodiesterase activity was inhibited about 60% when platelets were preincubated for 10 min with 100 μ M H-8, indicating that cAMP-dependent protein kinase was involved at some point in the stimulatory process. H-8 can also inhibit protein kinase C (30) but there is no evidence that cAMP can stimulate this enzyme.

Evidence for Phosphorylation of Low- K_m cAMP Phosphodiesterase. To test whether the stimulation of the low- K_m cAMP phosphodiesterase activity was accompanied by phosphorylation of the enzyme, platelets were preincubated with carrier-free 32 P $_4$ to label the internal adenosine nucleotides, including ATP, prior to treatment with forskolin. The 32 P-labeled platelets were then treated with forskolin and lysed, and the low- K_m cAMP phosphodiesterase was isolated by blue dextran-Sepharose chromatography. The fractions from the blue dextran-Sepharose column containing the low- K_m cAMP phosphodiesterase activity were pooled and run on NaDodSO $_4$ -containing polyacrylamide gels (19), and the gels were dried and analyzed by autoradiography. As shown in Fig. 4, incubation of platelets with forskolin resulted in a striking increase in the phosphorylation of a polypeptide of 110 kDa, the monomer molecular mass of the low- K_m cAMP phosphodiesterase.

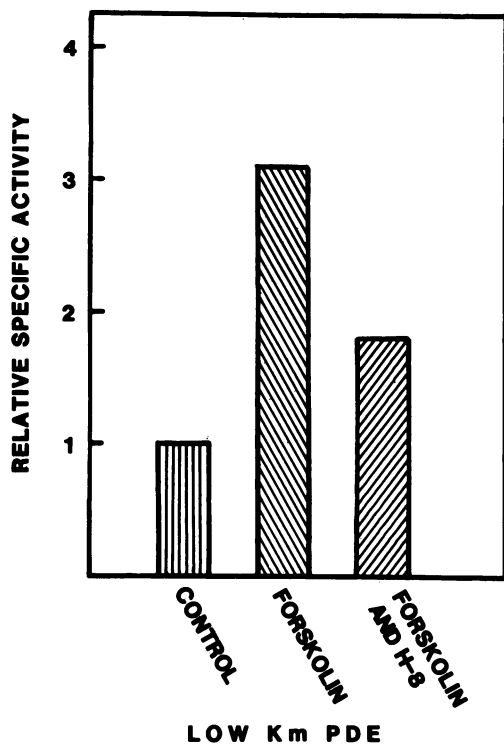


FIG. 3. Inhibition of forskolin stimulation of low- K_m cAMP phosphodiesterase (PDE) activity by the protein kinase inhibitor H-8. Platelets were preincubated for 10 min with 100 μ M H-8 before forskolin treatment. The low- K_m cAMP phosphodiesterase was purified by blue dextran-Sepharose chromatography. cAMP phosphodiesterase activity was measured at 1 μ M cAMP. Results are expressed as a ratio of the specific activity of the low- K_m cAMP phosphodiesterase from forskolin-treated or H-8- and forskolin-treated platelets to the specific activity of the phosphodiesterase from untreated platelets.

To determine if this labeled protein was the low- K_m cAMP phosphodiesterase, we prepared a nitrocellulose blot of a NaDodSO₄ gel of the low- K_m cAMP phosphodiesterase purified by blue dextran-Sepharose chromatography from ³²P-labeled, forskolin-treated platelets. When the blot was probed with the antiserum to the low- K_m cAMP phosphodiesterase,

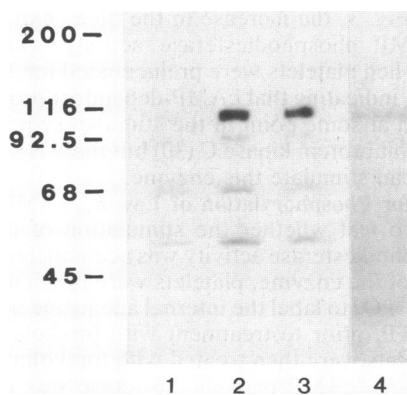


FIG. 4. NaDodSO₄/PAGE of purified low- K_m cAMP phosphodiesterase from platelets labeled with ³²P and treated with forskolin. Lanes 1–3 were derived from 5×10^9 platelets and lane 4 was derived from 10^{10} platelets. Lane 1, control, 11.5 μ g of protein; lane 2, 100 μ M forskolin, 5-min incubation, 12 μ g of protein; lane 3, 100 μ M forskolin, 10-min incubation, 10 μ g of protein; lane 4, 0.1 μ M iloprost, 4-min incubation, 18 μ g of protein. Following NaDodSO₄/PAGE, ³²P labeling was detected by autoradiography. Molecular masses are given in kDa.

a single band at 110 kDa, which comigrated with the ³²P label, reacted with the antiserum (Fig. 1, lanes 3 and 4).

To further test whether the labeled protein was the low- K_m cAMP phosphodiesterase, the antiserum was used in conjunction with fixed *S. aureus* cells to isolate the phosphodiesterase directly from a platelet lysate. Analysis of the immunoprecipitated protein by NaDodSO₄/PAGE revealed a major ³²P-labeled band at 110 kDa (Fig. 5), confirming that the stimulation of the low- K_m cAMP phosphodiesterase activity is accompanied by phosphorylation of the phosphodiesterase molecule.

Stimulation and Phosphorylation of Low- K_m cAMP Phosphodiesterase by Iloprost. Forskolin is a useful tool in probing cAMP-mediated intracellular regulation but is of course not present *in vivo*. In contrast, prostacyclin (prostaglandin I₂) is the major product of arachidonic acid metabolism produced by vascular cells and serves as a potent inhibitor of platelet function as well as a vasodilator under a variety of pathophysiological circumstances (31). Since prostacyclin has a short half-life in solution, we chose to use the stable analog iloprost (ZK 36374). Prostacyclin and iloprost activate adenylate cyclase by a receptor-linked mechanism utilizing the same receptor (32).

We therefore tested whether iloprost would also produce a stimulation of platelet low- K_m cAMP phosphodiesterase activity by phosphorylation of the enzyme. Iloprost, like prostacyclin, produces a transitory rise in intracellular levels of cAMP with the maximal rise occurring at 0.1 μ M iloprost after 4–5 min of incubation (33).

When platelets were incubated with iloprost, the activity of the low- K_m cAMP phosphodiesterase was stimulated 2.3-fold (± 0.6 -fold) and the enzyme was phosphorylated (Fig. 4). The level of stimulation of the low- K_m cAMP phosphodiesterase activity produced by iloprost was not as dramatic as that produced by forskolin. This difference is consistent with the fact that forskolin produces both a greater increase in and a more prolonged elevation of intracellular cAMP. These effects on cAMP levels should result in greater activation of the cAMP-dependent protein kinase and thus more dramatic activation of the low- K_m phosphodiesterase.

Subsequent to our initial report of these results (14), Macphee *et al.* (34) reported results of a similar experiment using platelets incubated with prostaglandin E₁. The level of stimulation of the low- K_m , cGMP-inhibited phosphodiesterase activity that they observed was less than that described here—i.e., 1.4-fold compared to 10-fold for forskolin or 2.3-fold for iloprost.

Harrison *et al.* (11) phosphorylated the cGMP-inhibited bovine phosphodiesterase by using a partially purified heart

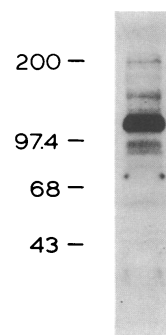


FIG. 5. Autoradiography of NaDodSO₄/polyacrylamide gel of the immunisolated low- K_m cAMP phosphodiesterase from forskolin-treated platelets. The low- K_m cAMP phosphodiesterase was isolated from a lysate prepared from 1.5×10^{10} platelets labeled with ³²P and treated with forskolin using *S. aureus* cells. Following NaDodSO₄/PAGE on a 7.5% gel, ³²P labeling was detected by autoradiography. Molecular masses are given in kDa.

kinase preparation as well as a purified cAMP-dependent protein kinase. However, they reported no change in the activity of the phosphodiesterase upon phosphorylation. This result is in marked contrast to the stimulation we observed when the low- K_m cAMP phosphodiesterase is phosphorylated in intact platelets. Whether this difference can be explained by phosphorylation of a different site in intact cells or whether phosphorylation is due to a different intracellular protein kinase is unknown at this time.

Our results support the hypothesis that the stimulation of phosphodiesterase activity in platelets following incubation of the cells with adenylate cyclase agonists is due to phosphorylation of the phosphodiesterase molecule. Phosphorylation may be a major mechanism for regulation of the activity of the low- K_m cAMP phosphodiesterase in platelets. Such activation of the phosphodiesterase following direct or receptor-mediated stimulation of the adenylate cyclase serves as a negative feedback modulating the responses of platelets to adenylate cyclase agonists. This increase in cAMP phosphodiesterase activity could play a major role in the down-regulation of platelets to further exposure to adenylate cyclase agonists.

Phosphorylation of other phosphodiesterases in purified or partially purified systems has been reported (11, 35–41). Different subunits of the purified Ca^{2+} /calmodulin-sensitive phosphodiesterase from bovine brain were phosphorylated by cAMP-dependent protein kinase and by a Ca^{2+} /calmodulin-sensitive protein kinase. These phosphorylations caused no change in the basal activity of the phosphodiesterase but decreased the binding affinity of the enzyme for calmodulin (37, 38). The plasma membrane phosphodiesterase in rat hepatocytes is stimulated when the cells are exposed to insulin in the presence of glucagon (35, 36). This activation appears to be due to a phosphorylation mechanism since the stimulation can be reversed when membranes isolated from treated cells are incubated with alkaline phosphatase (35, 36). The enzyme can also be directly phosphorylated in a purified membrane preparation by cAMP-dependent protein kinase, resulting in a stimulation of the phosphodiesterase activity (35, 36).

This low- K_m cAMP, cGMP-inhibited phosphodiesterase may have an important role in the regulation of other cells. This phosphodiesterase may play an important role in controlling cardiac muscle contractility and may be the target of a number of cardiotoxic drugs (11, 42). Such drugs are also potent inhibitors of platelet aggregation, suggesting that the low- K_m cAMP phosphodiesterase may be important in regulating platelet responses. The insulin-stimulated phosphodiesterase from rat adipocytes has been reported to be kinetically and immunologically similar to the low- K_m , cGMP-inhibited phosphodiesterase from bovine heart and human platelets (12, 43). The method of control of this enzyme is unknown but the maintenance of stimulation through partial purification suggests that the stimulation is due to a covalent modification of the enzyme, possibly phosphorylation (12).

The presence of a cGMP-inhibited phosphodiesterase as well as a cGMP-stimulated phosphodiesterase in platelets suggests that cGMP may play an important role in regulating cAMP metabolism. Since the K_m values of the two enzymes differ by an order of magnitude (9, 24) it is possible that cGMP regulates the basal and stimulated levels of cAMP. Recent reports that guanylate cyclase agonists inhibit platelet responses and increase cAMP levels in platelets suggest that the low- K_m , cGMP-inhibited phosphodiesterase may be inhibited by cGMP in intact cells and may play a role in the linkage of cAMP and cGMP metabolism in platelets (44–46).

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