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Immunological identification of the major platelet low-K_m cAMP phosphodiesterase: Probable target for anti-thrombotic agents

(cyclic nucleotides/proteolytic activation/immunoblots)

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ABSTRACT **Immunoblot and enzyme-activity analyses, using specific immunological probes, indicated that more than 80% of the total low-K_m cAMP phosphodiesterase activity present in bovine and human platelets resided in a single phosphodiesterase isozyme. In the presence of protease inhibitors, the platelet enzyme has an apparent subunit size of 110 kDa and appears immunologically and structurally indistinguishable from a recently purified bovine heart isozyme. When protease inhibitors were absent during homogenization and centrifugation, this platelet phosphodiesterase was susceptible to sequential proteolysis forming 80-kDa and 60-kDa peptides. As a previous report on the purification of the platelet low-K_m cAMP phosphodiesterase described a 61-kDa protein, our data would suggest that this was a proteolytic fragment. Moreover, in our study a 40–70% increase in catalytic activity was associated with proteolysis. Further similarities between the platelet and heart phosphodiesterases were demonstrated by pharmacological studies that showed identical inhibitor profiles for both enzymes. Several known phosphodiesterase inhibitor compounds that have been found useful in inhibiting platelet aggregation also inhibited the platelet low-K_m cAMP phosphodiesterase with potencies very similar to their antithrombotic effects. Cilostamide, Ro 15-2041, milrinone, papaverine, isobutylmethylxanthine, and theophylline inhibited the 110-kDa platelet enzyme with IC_{50} values of 0.04, 0.13, 0.46, 1.4, 2.6, and 110 μM, respectively.**

The hydrolysis of the important cytosolic "second messenger" molecules, cAMP and cGMP, is now known to be carried out by several phosphodiesterase isozymes. These differ from one another in terms of their regulation, substrate affinities, and rates of hydrolysis (1). Platelets have been reported to contain three different phosphodiesterase forms (2, 3): the first was relatively specific for cGMP, whereas the second appeared to be nonselective. The third, however, seemed to preferentially hydrolyze cAMP and has been reported in its purified form to be a low-K_m enzyme of 61-kDa by $NaDodSO_4$/PAGE analysis (4).

Since platelet responsiveness appears to depend on a balance between signal-transducing pathways that lead to changes in cytosolic free Ca^{2+} or cAMP (5), the regulation of cAMP levels is crucial to the control of platelet function. Calcium-mobilizing agonists (e.g., thrombin or thromboxane A_2) promote platelet activation, whereas agents that increase cellular cAMP concentrations (e.g., prostacyclin or prostaglandin E_1) act to inhibit platelet function (5, 6). This opposing effect of elevated cAMP levels has prompted investigation into the development of compounds that could selectively inhibit platelet cAMP phosphodiesterase activity and act as antithrombotic agents. Cilostamide (3) and Ro 15-2041 (7) are two potent inhibitors of platelet aggregation and have been shown recently to be equally effective inhibitors of the platelet low-K_m cAMP phosphodiesterase (the so-called "F III phosphodiesterase"; ref. 3).

A major goal of many investigators has been the purification of this cAMP phosphodiesterase present in platelets. This would lead to a greater understanding of how the enzyme functions and allow the production of specific monoclonal antibodies, probes that would allow further characterization of the isozyme. Such information would undoubtedly aid the formulation of selective inhibitors for possible use as antithrombotic agents.

A novel low K_m cAMP phosphodiesterase isozyme has been purified in our laboratory from bovine heart (8). This enzyme has been designated as cGMP-inhibited phosphodiesterase, since low concentrations of cGMP inhibit ($K_i = 6 \times 10^{-8}$ M) cAMP hydrolysis. This paper demonstrates, through immunological, physical, and pharmacological studies, that the platelet low-K_m cAMP phosphodiesterase appears identical to the recently purified isozyme from bovine heart. The implications of this finding are discussed with regard to the cellular regulation of the enzyme and its role in the mechanism of action of antithrombotic agents in the treatment of ischemic heart disease.

MATERIALS AND METHODS

Materials. Milrinone was a gift from Sterling–Winthrop Research Institute, and cilostamide was kindly provided, in absolute concentration, by H. Hidaka (Department of Pharmacology, Mie University, Edsbashi, Japan). Ro 15-2041 was generously supplied by R. Muggli (Pharmaceutical Research Department, Hoffmann–La Roche, Basel, Switzerland). Papaverine, isobutylmethylxanthine, cGMP, and theophylline were all purchased from Sigma.

Platelet Preparation and Fractionation. Bovine platelet suspensions were prepared from 800 ml of fresh blood collected in 200 ml of buffer containing 1.33 g of EDTA, 0.9% saline, and 2 g of glucose (pH 7.4). This was centrifuged at 900 $\times$ *g* for 6 min at room temperature, and the resulting platelet-rich plasma was recentrifuged at 400 $\times$ *g* to remove contaminating leukocytes and erythrocytes. Platelets were then sedimented at 2200 $\times$ *g* for 9 min and washed three times by resuspension/centrifugation in isotonic Tris-buffered saline (pH 7.4) containing 135 mM NaCl, 20 mM Tris·HCl, 13 mM sodium citrate, 5 mM glucose, and 2 mM EDTA. The resulting bovine platelet pellet (0.4–0.5 g wet weight) was resuspended in 7 ml of homogenization buffer (40 mM Tris·HCl, pH 7.8/15 mM 2-mercaptoethanol/50 mM benzamidine·HCl/20 μg of leupeptin per ml at 4°C) and homogenized by sonication (Branson Sonic Power sonifier, 65% intensity) twice for 15 sec. In some experiments the platelet pellet was split into two portions and homogenized in 3.5 ml of buffer with or without benzamidine and leupeptin. Homogenates were centrifuged at 40,000 $\times$ *g* for 30 min at 4°C in a Beckman 75 Ti ultracentrifuge rotor. The resulting pellet was washed once by resuspension/centrifugation in 10 ml of

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homogenization buffer and finally resuspended in the original supernatant volume.

Human platelets were obtained from healthy donors and prepared by the method of Ardlie *et al.* (9). Platelets were washed once with the wash buffer described above before homogenization (5×10^9 per ml) and centrifugation as outlined for bovine platelets.

DEAE-Cellulose Chromatography. A 5-ml fraction of bovine platelet supernatant from centrifugation at 40,000 × *g* was applied to a 2.5-ml DEAE-cellulose column equilibrated in 50 mM Tris·HCl, pH 7.8/2 mM EDTA/15 mM 2-mercaptoethanol/0.1 M NaCl. The column was then washed with 20 ml of the same equilibrating buffer. Elution was performed with a 10-ml linear NaCl gradient (0.1–0.7 M), and 0.5-ml fractions were collected.

Immunoadsorption and Immunoblot Analysis. Solid-phase antibody reagents (Pansorbin-based) were prepared by using a specific monoclonal antibody (CGI-5; ref. 8) directed against the cGMP-inhibited phosphodiesterase from bovine heart. These procedures have been described in detail (8, 10). A monoclonal antibody (ROS-1; ref. 11) directed against a phosphodiesterase isolated from the bovine rod outer segment, but of the same immunoglobulin subtype as CGI-5, was used to measure nonspecific adsorption of platelet supernatant phosphodiesterase activity. Such adsorption was equivalent or less than adsorption produced with solid-phase reagent in the absence of primary antibody (data not shown).

For immunoblot analysis, platelet pellet or supernatant fractions were boiled in an equal volume of sample buffer containing $NaDodSO_4$. Samples were analyzed by $NaDodSO_4$/PAGE on a 10% gel (12). Protein transfer to nitrocellulose and subsequent immunoblotting procedures have been described elsewhere (8). Controls were performed with preimmune sera or control monoclonal antibody and produced essentially blank autoradiograms (data not shown).

Phosphodiesterase Assay and Inhibitor Study. Phosphodiesterase activity was assayed at 30°C as described (10). Rates of hydrolysis were linear over the range of times and dilutions used. Monoclonal antibody–phosphodiesterase complexes were prepared from platelet supernatants and used in inhibitor studies. Such complexes have been shown not to interfere with phosphodiesterase catalytic activity and, therefore, are ideal for conducting inhibitor studies against one specific enzyme (8, 10). Control experiments demonstrated that the drug vehicles did not interfere with the phosphodiesterase assay and also that none of the inhibitors tested affected snake venom activity necessary for the assay. Finally, protein determinations were performed by the method of Bradford (13) with bovine serum albumin (Sigma) as standard.

RESULTS

Identification of the Phosphodiesterase as a 110-kDa Protein. When bovine platelet cAMP phosphodiesterase activity was measured at 1 μM substrate after homogenization and centrifugation, ≈85% of the activity was found in the supernatant at 40,000 × *g* as compared to ≈15% in the pellet (Fig. 1*A*). Almost 90% of this supernatant activity was specifically immunoadsorbed after incubation with a solid-phase monoclonal antibody (CGI-5; ref. 8) directed against a bovine heart low-K_m cAMP phosphodiesterase (Fig. 1*B*).

Immunoblot analysis of the bovine platelet supernatant with CGI-5 monoclonal antibody showed that the phosphodiesterase had an apparent subunit molecular mass of 110 kDa (Fig. 1*C*, lane 1). This was identical to that of the bovine heart enzyme (Fig. 1*C*, lane 5). A quantitatively much weaker 110-kDa signal was observed from the immunoblot of the particulate fraction (Fig. 1*C*, lane 2), suggesting that at least a portion of the activity associated with the pellet was

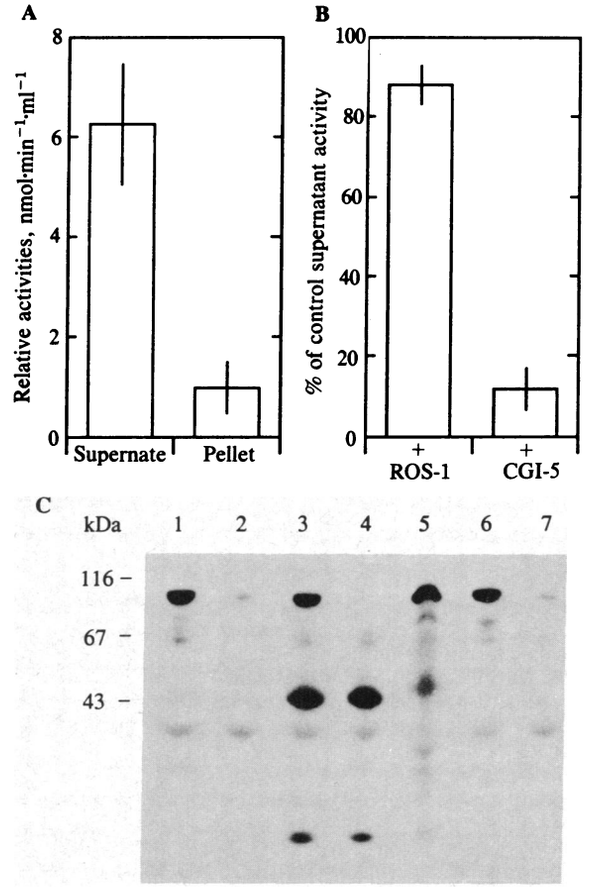

FIG. 1. Activity and immunoblot analysis of platelet low-K_m cAMP phosphodiesterase. Platelet fractionation, immunoadsorption studies, and protein immunoblot analysis were performed as described. Phosphodiesterase activity was measured by using 1 μM cAMP. (*A*) Relative activities present in supernatant versus resuspended pellet. (*B*) Specific immunoadsorption of supernatant activity by CGI-5 versus ROS-1 monoclonal antibodies. (*C*) Immunoblot analysis with CGI-5. Lanes: 1, bovine platelet supernatant; 2, bovine platelet pellet; 3, bovine platelet supernatant after immunoadsorption with ROS-1; 4, bovine platelet supernatant after immunoadsorption with CGI-5; 5, bovine heart extract; 6, human platelet supernatant; 7, human platelet pellet. Note the presence of contaminating immunoglobulin heavy and light chains in lanes 3 and 4. The specific activities (at 1 μM cAMP) of bovine and human platelet supernatants were 1.2 ± 0.4 and 0.8 $nmol \cdot min^{-1} \cdot mg^{-1}$, respectively.

contributed by the 110-kDa protein. Lanes 3 and 4 in Fig. 1*C* show that a close correlation existed between specific immunoprecipitation of supernatant activity by CGI-5 and a reduction in the 110-kDa blotting signal.

Human platelets were similarly fractionated and subjected to the same analyses. In addition to identifying the phosphodiesterase as a 110-kDa peptide, the percentage of total activity that was soluble and the specific immunoadsorption of activity by CGI-5 were identical for both bovine and human platelets (Fig. 1*C*, lanes 6 and 7; also data not shown).

Bovine platelet supernatant was applied to a DE-52 cellulose column and eluted with a linear NaCl gradient; fractions were assayed for cAMP phosphodiesterase at 1 μM substrate (Fig. 2). The profile obtained was similar to those previously reported (3, 4), since only one major peak of activity was observed at low concentrations of substrate. A similar profile was found after immunoblot analysis of each of the fractions (Fig. 2 *Inset*) in that the 110-kDa protein signal was most intense in the same fractions as was the major peak of phosphodiesterase activity. This was verified quantitatively

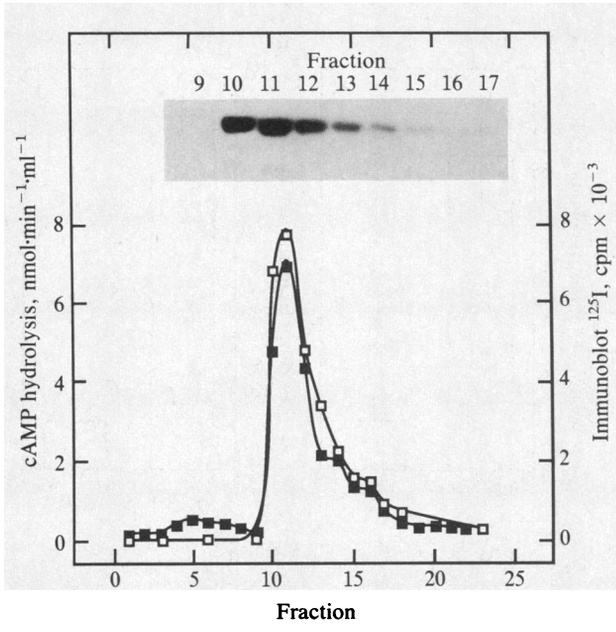

FIG. 2. DEAE-cellulose chromatography of bovine platelet supernatant from centrifugation at 40,000 × *g*. Fractions (0.5 ml) were eluted with a linear 0.1–0.7 M NaCl gradient and either assayed at 1 μM cAMP (■) or subjected to protein immunoblot analysis with CGI-5 (*Inset*). ^{125}I radioactivity was also measured from each lane of the immunoblot (□).

by counting the level of ^{125}I radioactivity associated with each lane. The peak of radioactivity was found to completely overlap with the peak of cAMP phosphodiesterase activity (Fig. 2). These results indicated that more than 90% of the platelet supernatant low-K_m cAMP phosphodiesterase activity was associated with the 110-kDa protein.

Proteolytic Degradation. The susceptibility of the 110-kDa peptide from bovine platelets to proteolytic degradation to two smaller fragments of around 80 kDa and 60 kDa is shown in Fig. 3. This was achieved by omission of protease inhibitors during homogenization and centrifugation. Supernatant samples were boiled in sample buffer immediately after centrifugation (zero time) or after incubation at room temperature for 3 or 6 hr. An immunoblot study was then performed with specific mouse antiserum (10) to the bovine

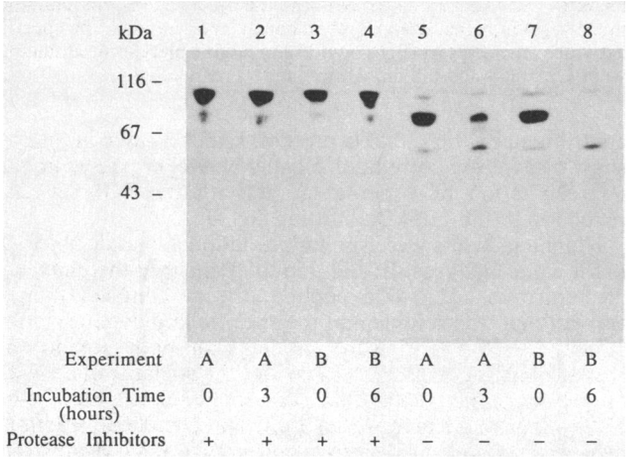

FIG. 3. Demonstration of phosphodiesterase proteolysis by protein immunoblot analysis. Bovine platelets from two different preparations were homogenized/centrifuged in the presence (lanes 1–4) or absence (5–8) of protease inhibitors (i.e., 50 mM benzamidine·HCl and 20 μg of leupeptin per ml). Immunoblot analysis with mouse antiserum was performed after incubation at room temperature for the indicated periods of time.

heart enzyme instead of CGI-5, since the former did not recognize the 60-kDa protein (data not shown). In the presence of protease inhibitors, the only major signal at all time points studied was the 110-kDa peptide (Fig. 3, lanes 1–4). However, in the absence of protease inhibitors, the immunoblot pattern was significantly altered (Fig. 3, lanes 5–8). Even at zero time, marked proteolysis was evident with the appearance of two fragments at 80 kDa and 60 kDa (Fig. 3, lanes 5 and 7). Most of the reduction in the 110-kDa signal at this time point seemed to be recovered in the 80-kDa band. At longer incubations, the intensity of the 60-kDa signal was further increased (Fig. 3, lanes 6 and 8). After 6 hr of incubation, the intensity of the total signal was reduced, suggesting breakdown to even smaller fragments, which were not recognized by the antiserum. Finally, it should be noted that concomitant with proteolysis at zero time was a 40–70% increase in low-K_m cAMP phosphodiesterase activity.

Inhibitor Studies. Table 1 shows IC_{50} values for the inhibition of bovine low-K_m cAMP phosphodiesterase by several inhibitors of phosphodiesterase. Cilostamide, Ro 15-2041, cGMP, and milrinone were all potent inhibitors of platelet cAMP phosphodiesterase with IC_{50} values of 0.04, 0.13, 0.13, and 0.46 μM, respectively. In contrast, both theophylline and Ro 20-1724 were more than 2 orders of magnitude less potent. Furthermore, the values given for cGMP, milrinone, papaverine, isobutylmethylxanthine, and Ro 20-1724 are in close agreement with inhibitor data obtained with the bovine heart isozyme (8, 10).

DISCUSSION

The data presented in this study demonstrated that the low-K_m cAMP phosphodiesterase present in platelets appears similar or identical to a recently purified 110-kDa isozyme from bovine heart (8, 10). In addition to the appropriate platelet activity being specifically immunoadsorbed by a monoclonal antibody directed against the bovine heart enzyme, the phosphodiesterase activity was clearly associated with a 110-kDa protein. This peptide was found to be very susceptible to proteolytic activation as a result of degradation to two major fragments of ≈80 kDa and ≈60 kDa. Interestingly, the platelet low-K_m cAMP phosphodiesterase has been reported as a purified enzyme of 61 kDa (4). Our data would suggest that this 61-kDa protein was a proteolytic fragment of the 110-kDa form.

A phosphodiesterase inhibitor study was conducted to compare known inhibitor data for the bovine heart enzyme (8, 10) to values obtained with monoclonal antibody-purified cAMP phosphodiesterase from bovine platelets. The values obtained in this study were in close agreement to those previously reported for the heart enzyme. Low concentrations of cGMP inhibited the hydrolysis of cAMP by the platelet enzyme (IC_{50} = 0.13 μM), supporting the designation of such activity as "cGMP-inhibited" (8, 10). The new

Table 1. Inhibitor values for the platelet low-K_m cAMP phosphodiesterase

Inhibitor	IC_{50}, μM
Cilostamide	0.04 ± 0.01
cGMP	0.13 ± 0.02
Ro 15-2041	0.13 ± 0.02
Milrinone	0.46 ± 0.06
Papaverine	1.40 ± 0.26
Isobutylmethylxanthine	2.60 ± 0.12
Theophylline	110.00 ± 15.00
Ro 20-1724	220.00 ± 64.00

Values were calculated from 3 or 4 separate determinations with CGI-5-purified enzyme and 0.35 μM cAMP as substrate. Data are the means ± SD.

cardiotonic drug milrinone, previously shown to be ≈100-fold selective for the bovine heart cAMP phosphodiesterase when compared with two other purified isozymes (10), was equipotent in inhibiting the platelet enzyme. Isobutylmethylxanthine and papaverine, two relatively nonselective inhibitors (8), were found to be potent inhibitors of platelet cAMP phosphodiesterase. In contrast, both theophylline and Ro 20-1724 were poor inhibitors. It is noteworthy that these inhibitor data are in close correlation with the relative potencies of cilostamide, Ro 15-2041, isobutylmethylxanthine, papaverine, and theophylline in inhibiting platelet aggregation (3, 7, 14, 15).

The finding that two potent inhibitors of platelet aggregation, cilostamide and Ro 15-2041, were also potent inhibitors of platelet cAMP phosphodiesterase (refs. 3 and 7 and this study) is of some interest. First, both these drugs, like milrinone (16), have stimulatory effects on myocardial contractility (7, 17). Second, from the results presented in this paper, milrinone, like cilostamide and Ro 15-2041, would be expected to be a potent inhibitor of platelet aggregation. Third, a close connection is beginning to emerge between antithrombotic agents, which are cAMP phosphodiesterase inhibitors, and their use in the treatment of ischemic heart disease (18, 19). It is tempting to speculate that the locus of such an action lies at the level of cGMP-inhibited phosphodiesterase.

It has been demonstrated that neither cilostamide nor Ro 15-2041 is able to increase basal cAMP concentrations in platelets. However, both were able to inhibit agonist-induced aggregation and potentiate the increase in platelet cAMP concentration stimulated by prostacyclin or prostaglandin E_1 (3, 7). These data support the idea that two pools of cAMP exist in platelets (15). One of these is thought to be a small functional pool that is sensitive to the action of low-K_m cAMP phosphodiesterase inhibitors. A similar observation was noted with adipocytes, where cilostamide was found to promote lipolysis (20). In this particular study, cilostamide was used to selectively inhibit the insulin-sensitive low-K_m cAMP phosphodiesterase. This adipocyte phosphodiesterase is also inhibited by low concentrations of both cGMP and milrinone and, interestingly, has been immunoadsorbed by antiserum to the bovine heart low-K_m isozyme (21).

Finally, incubation of intact platelets with either prostacyclin or prostaglandin E_1 leads to an activation of low-K_m cAMP phosphodiesterase activity (22, 23). This activation has been postulated as a negative-feedback regulation of agonist-induced increases in cAMP levels. Furthermore, some findings suggest that activation of a cAMP-dependent protein kinase is an intermediate step (23, 24).

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