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Effect of 2-(1-piperazinyl)-4H-pyrido[1,2-a]pyrimidin-4-one (AP155) on Human Platelets *In Vitro*

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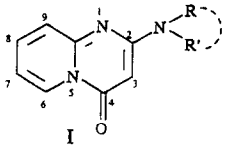
ABSTRACT. The effect on human platelets of 2-(1-piperazinyl)-4H-pyrido[1,2-a]pyrimidin-4-one (AP155) was tested *in vitro* by measuring cyclic adenosine monophosphate (cAMP) level, cytosolic Ca^{++}, [^{125}I]fibrinogen binding as well as aggregation induced by several agonists. AP155 dose-dependently inhibited aggregation both in platelet rich plasma (PRP) and in washed platelets (WP), exerting its maximal power in the presence of collagen, ADP and platelet activating factor (PAF). It specifically inhibited the activity of cAMP high affinity phosphodiesterase (PDE), resulting in a sufficient increase in cAMP levels to activate cAMP-dependent protein kinase. AP155 was able to inhibit aggregation, the increase in cytosolic Ca^{++} induced by thrombin, and fibrinogen binding to ADP or thrombin-stimulated platelets. Thus, this new pyridopyrimidine derivative exerts its antiplatelet activity by increasing cAMP intracellular concentration. BIOCHEM PHARMACOL **53**;11: 1667–1672, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Pyridopyrimidine derivative; antiplatelet activity; mode of action; cAMP; cAMP phosphodiesterase

In recent papers [1, 2] we described the synthesis and platelet aggregation inhibitory properties of 2-(dialkylamino)-4H-pyrido[1,2-a]pyrimidin-4-ones I, and of their angular isomeric 6,7 or 8,9 benzo-fused derivatives II and III, respectively.



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A number of compounds I–III of these series were tested *in vitro* for their inhibitory activity on the aggregation of human platelets induced in platelet rich plasma (PRP) by ADP, collagen, or the Ca^{++} ionophore A23187 [1, 2]. The results obtained allowed us to draw some structure-activity relationship conclusions, among which the most significant are:

a) Between the two tricyclic structures II and III, the former appears the most convenient for antiplatelet activity, with compounds II showing inhibitory properties on human platelet aggregation nearly equivalent to those exhibited by the corresponding bicyclic compounds I, which on the whole are the most active;
b) Among the dialkylamino substituents used, 1-piperazinyl produces the highest activity towards all the platelet aggregation inducers used;
c) The 1-piperazinyl substituted compound 2-(1-piperazinyl)-4H-pyrido[1,2-a]pyrimidin-4-one (AP155) (series I, Fig. 1) is the most active of all the compounds tested and can be regarded as a very interesting *in vitro* antiplatelet agent.

The novelty of the AP155 structure, with respect to this biological field, has now prompted us to investigate the mode of action of this compound.

We present here the *in vitro* effects of AP155 on human platelets, evaluated through the aggregation assay both in PRP and washed platelets (WP), measurements of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels and of cAMP protein kinase activity, studies of platelet phosphodiesterase (PDE) enzymes, and the effects on [Ca^{++}] transients and on [^{125}I]fibrinogen binding. The results obtained indicate that the drug selectively inhibited cAMP high affinity PDE activity, with a detectable increase in platelet cAMP levels and consequent activation of cAMP protein kinase. Aggregation, cytosolic Ca^{++} increase and fibrinogen binding were also inhibited.

MATERIALS AND METHODS

Chemicals

[^{3}H]cAMP, [^{3}H]cGMP, Iodine-125, [γ-^{32}P]ATP, cAMP and cGMP radioimmunoassay kits were from Amersham

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Abbreviations: AP155, 2-(1-piperazinyl)-4H-pyrido[1,2-a]pyrimidin-4-one; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; IBMX, 3-isobutil-1-methylxantine; LDH, lactate dehydrogenase; PAF, platelet activating factor; PDE, phosphodiesterase; PMA, phorbol myristate acetate; PRP, platelet rich plasma; WP, washed platelets.

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(Little Chalfont, England). Thrombin, phorbol myristate acetate (PMA), Ca^{++} ionophore A23187, 3-isobutyl-1-methylxantine (IBMX), aequorin, ADP, ATP and GTP were from Sigma Chemical Co. (St. Louis, MO, USA). Human fibrinogen was from Protogen AN (Laufelfingen, Switzerland) and Iodo-Beads Iodination reagent from Pierce (Rockford, IL, USA). Platelet activating factor C18 (PAF) was from Novabiochem AG (Laufelfingen, Switzerland) and collagen from Mascia Brunelli (Milan, Italy). The stable PGI_2 analogue Iloprost was a gift from Schering AG (Berlin, Germany). PMA and PAF stock ethanol solutions were further diluted in saline. A23187 was stocked in DMSO and diluted in saline. AP155 was prepared as previously described [2].

Statistics

Unless otherwise indicated all results are means ± SD of four experiments carried out in duplicate. In all cases similar findings were obtained in a minimum of three experiments. A control experiment in the presence of solvent was always carried out. Statistically significant differences were determined by using Student's two-tailed unpaired *t*-test.

Human Platelet Preparation and Aggregation

Human blood obtained from healthy volunteers was collected in 130 mM Na-citrate (9:1). PRP and WP were prepared as previously described [3]. Aggregation, performed on an Aggrecoder PA-3210 Menarini aggregometer (Florence, Italy), was measured following the turbidimetric method of Born [4] and quantified by the light transmission reached within 3 min. PRP and/or WP were preincubated for 2 min at 37°C with vehicle or drug solution before addition of the agonist. Treatment of platelets with the drug did not produce an increase in lactate dehydrogenase (LDH) release as compared to untreated cells. In both cases the LDH release corresponded to 10% of the total enzymatic activity.

cAMP Measurements

PRP was preincubated for 5 min at 30°C with either solvent or the test compound solution, then iloprost was added when required. The reaction was stopped after 5 min by the addition of cold 0.7 M perchloric acid. The mixtures were sonicated for 5 sec on ice, then centrifuged for 2 min at 12000 g. Supernatants were neutralized with 2M NaOH and immediately analysed for cAMP content by radioimmunoassay.

Determination of Cyclic Nucleotide PDE Activity

Platelet soluble cyclic nucleotide PDE activity was measured as previously reported [5].

cAMP-dependent Protein Kinase Assay

WP (5.0×10^8/mL) were incubated at 37°C with vehicle or drug solution for the described time (Fig. 5). At the end of incubation, suitable aliquots of the control and drug-treated WP were added to equal volumes of ice-cold lysing buffer (pH 6.8) consisting of 10 mM EDTA, 0.5 mM IBMX, 0.2% Triton X-100, and 10 mM KH_2PO_4. The samples were quickly vortex-mixed, then immediately immersed in dry ice and stored at −80°C until use. cAMP-dependent protein kinase was assayed according to Seiler *et al.* [6]. Activity was measured in a medium containing 60 mM 3-(N-morpholino)-propanesulfonate, 15 mM $MgCl_2$, 2.5 mM EGTA, 0.32 mg/mL BSA, 125 μM Kemptide and 125 μM ATP (approximately 450 cpm/pmol of [γ-^{32}P] ATP), with or without 10 μM cAMP.

Thrombin-Stimulated Calcium Mobilization

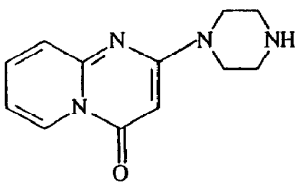
WP were loaded with the photoprotein aequorin according to Yamaguchi *et al.* [7]. The increase in cytosolic Ca^{++} following platelet stimulation with thrombin was quantified by the peak luminescence reported by aequorin and compared to the peak luminescence obtained on lysis of a separate aliquot of platelets by 0.1% Triton X-100. Luminescence was quantified with a PICA Lumiaggregometer (Chronolog Corp., Philadelphia, PA, USA).

Fibrinogen Iodination

Ten μL of human fibrinogen solution (1 mg/mL) was mixed with 100 μL of 0.2 M phosphate buffer (pH 7.5), 270 μCi of [^{125}I]Iodide and one Iodo-Bead. After 10 min at room temperature, the reaction was stopped by removing the bead. [^{125}I]fibrinogen was separated from free iodide using a Sephadex G-10 (Sigma Chemical Co., St. Louis, MO, USA) column equilibrated with PBS containing 1% of BSA. Fibrinogen was iodinated to achieve specific radioactivity of 2–5 × 10^9 cpm/mg. It was stored in a freezer at −20°C and used for 14 days.

[^{125}I]fibrinogen Binding Studies

Blood collected from human volunteers into ACD (9:1) was centrifuged at 100 g for 10 min. PRP was added to apyrase (1 U/mL) and centrifuged, then the pellet was washed once and resuspended in Tyrode's-HEPES buffer (pH 7.5) containing 2.0 mM Ca^{++}. The pooled platelets (5.0×10^8 plts/mL) were activated by 20 μM ADP or 0.1 U/mL thrombin for 5 min at room temperature, then mixed with various concentrations of [^{125}I]fibrinogen (2.0×10^5 cpm/μg). After 15 min incubation at 25°C, suitable aliquots were centrifuged down through a 20% w/v sucrose cushion. The supernatant was tested for free radioactivity while bound radioactivity was measured in the platelet pellet cut off together with the tip of the tube counting in the gamma counter (Packard Instruments Co. Meriden,



AP 155

FIG. 1. 2-(1-Piperazinyl)-41·1-pyridol[1,2-a]pyrimidin-4-one.

CT, USA). After stimulation with thrombin, the enzymatic activity of this inducer was blocked by hirudin (0.3 U/mL). To examine the effect of AP155, platelet suspensions were preincubated for 3 min with AP155 before addition of the other agents. Non-specific binding, measured in the presence of 10 mM EGTA and 2.5 mg/mL unlabeled fibrinogen, was 10–15% of the total bound fibrinogen. Values of fibrinogen specific binding, obtained from three separate experiments carried out in duplicate, were analysed according to Scatchard [8] and the number of binding sites and dissociation constant (K_d) were calculated.

RESULTS

Incubation of PRP for 2 min at 37°C with AP155 (Fig. 1) produced inhibition of platelet aggregation. AP155 inhibited platelet aggregation induced by collagen, ADP and PAF with almost equal potency, the IC_{50} values being 3.6 ± 1.2, 6.0 ± 1.8, 7.5 ± 1.4 μM respectively. AP155 was less effective in the presence of A23187 and PMA both in PRP and in WP (Table 1). The similarity of IC_{50} values in WP and PRP indicates the low binding of AP155 to serum proteins. In some experiments PRP was treated with aspirin and then challenged by collagen and ADP. Since the inhibition in PRP and aspirin-treated PRP was similar, it is likely that AP155 does not affect the cyclooxygenase pathway and consequently endogenous thromboxane A_2 formation.

The action of compounds active against all agonists includes an increase in cAMP concentration and the blockade of glycoprotein IIb-IIIa receptors. To determine the mechanism of action of AP155, we measured the cAMP levels in PRP and WP in the presence and absence of AP155. As with other compounds described in the literature [6, 9], AP155 slightly increased the cAMP basal level in resting platelets. Nevertheless, it potentiated the ability of adenylate cyclase activators such as iloprost to increase cAMP. The effect was dose-dependent and comparable to that of IBMX (Fig. 2). Similar results were obtained in PRP and in WP. Furthermore AP155 did not modify cGMP basal levels: 1.12 ± 0.07 and 1.0 ± 0.06 pmol × 10^8 platelets were measured with and without 100 μM AP155, respectively.

TABLE 1.

Agonist	PRP	WP
	IC_{50} (μM)	
ADP (5 μM)	6.0 ± 1.8	ND
A23187 (20 μM)	19.0 ± 9.0	19.3 ± 10.0
Collagen (5 μg/mL)	3.6 ± 1.2	ND
PAF (1 μM)	7.5 ± 1.4	ND
PMA (1 μM)	28.8 ± 9.6	35.0 ± 8.8
Thrombin (0.1 U/mL)	ND	29.3 ± 5.0

Inhibition of human platelet aggregation by AP155.

PRP or WP (2.0×10^8/mL) were preincubated with solvent or drug solution for 2 min at 37°C and then challenged with agonist. Values are mean ± SD of at least six separate determinations. ND: not determined.

As the increase in cAMP was either due to adenylate cyclase activation or PDE inhibition, the influence of AP155 on these enzymes was studied. Since no effect of AP155 on adenylate cyclase activity was demonstrated, it was reasonable to suppose that AP155 may be a PDE inhibitor. To verify this hypothesis, we assayed the effect of AP155 on PDE activity both on a crude preparation and on the soluble fraction of human platelets in the presence of 1 or 10 μM cAMP. The IC_{50} values obtained in the soluble fraction were 19 ± 6 μM or 284 ± 46 μM and those from crude preparation 21 ± 5 or 279 ± 38 μM respectively. Fig. 3 shows a dose-response curve of the AP155 inhibitory effect on soluble high affinity cAMP-PDE. It is remarkable that the rank order of AP155 inhibitory potency versus PDE was the same as that observed when the compound was used to inhibit platelet aggregation. AP155 poorly inhibited cGMP-PDE activity (Fig. 3).

Among the ways in which cAMP inhibits platelet function is by reducing signal transduction and Ca^{++} mobilization. We therefore examined the effect of AP155 on thrombin-stimulated increases in cytosolic Ca^{++} of aequorin-loaded WP. AP155 was able to almost completely inhibit calcium elevation as well as aggregation evoked by thrombin. The dose-response curves for both inhibition of calcium mobilization and that of platelet aggregation were similar. Calculated IC_{50} values were 21.6 μM and 28.8 μM for the Calcium response and platelet aggregation respectively (Fig. 4).

Small changes in platelet cAMP levels, even elevations less than 2-fold those produced by AP155, were sufficient to activate most of the cAMP protein kinase, as shown in Fig. 5. The activity was measured by the cAMP-dependent protein kinase ratio, defined as the fraction of the total cell cAMP-dependent protein kinase activated by endogenous cellular cAMP. It was determined as the ratio of the phosphorylation of peptide substrate by cell sonicate measured without cAMP addition to that measured after excess cAMP addition. AP155 had no effect on protein kinase if platelets were sonicated before drug addition. Since changes in cAMP levels regulate fibrinogen receptor exposure on human platelets, we wanted to verify if AP155 could modify fibrinogen binding. Before examining the effect of AP155, we established the characteristics of [^{125}-I]fibrinogen binding to human platelets. In ADP-treated platelets, the number of fibrinogen binding sites per platelet was 56500 ± 7400 and the K_d value 0.12 ± 0.01 μM. After stimulation with thrombin, 54500 ± 6000

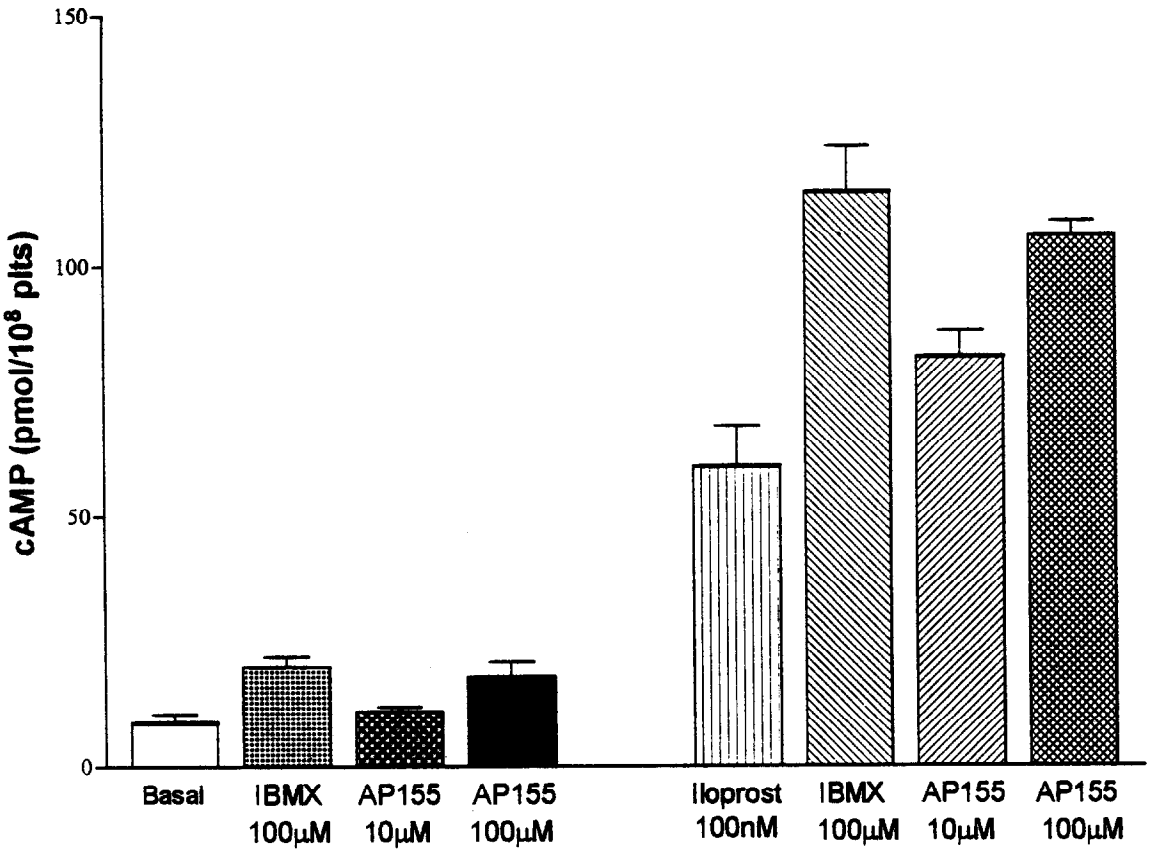


FIG. 2. Effect of AP155 on cAMP levels. Human PRP was incubated for 5 min at 30°C with solvent or the test compound, then 100 nM iloprost was added when required. After 5 min at 30°C, the reaction was stopped and cAMP measured. Each bar represents the mean ± SD of three experiments carried out in duplicate.

molecules of [^{125}I]fibrinogen per platelet were bound and the K_d was 0.26 ± 0.05 μM. We next examined the effect of AP155 on fibrinogen binding. As shown in Fig. 6, AP155 inhibited both ADP and thrombin-induced mobilization of binding sites for fibrinogen: the IC_{50} value (27 ± 3 μM) for ADP-activated platelets did not significantly differ from the IC_{50} value (19 ± 2 μM) measured for thrombin-treated platelets.

DISCUSSION

Platelet activation is one of the first steps in the development of thrombosis and atherosclerosis. Platelets are involved in the thrombotic complications that lead to myocardial infarction and occlusive stroke and are important for the initiation of lesions of the vascular wall and stimulation of smooth muscle cell proliferation [10, 11]. Because of the critical role of platelets, considerable effort has been expended toward the discovery and clinical study of antiplatelet drugs. Drugs that can inhibit platelet activation in response to all agonists without inducing side-effects should be very useful. One of the best ways to inhibit platelet function is to increase intracellular cAMP levels. cAMP inhibits platelet activation at several steps, one of the most important being the inhibition of receptor-mediated phosphoinositide hydrolysis by phospholipase C

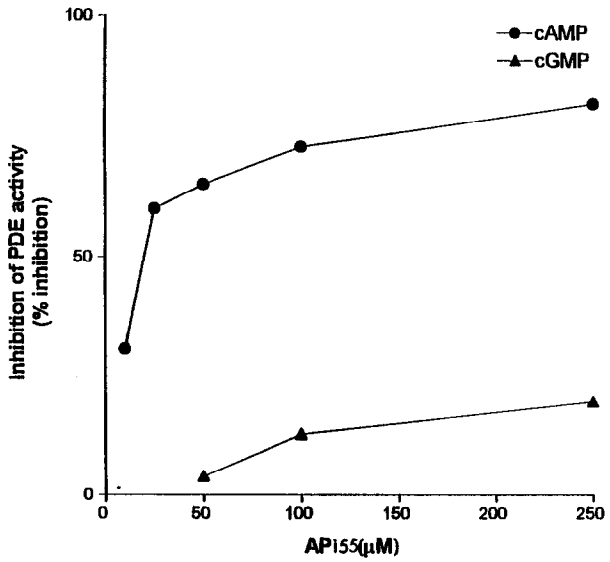


FIG. 3. Effect of increasing amounts of AP155 on soluble cyclic nucleotide PDE. Cytosolic fraction preincubated for 10 min at room temperature with solvent or AP155 was incubated for 10 min at 30°C with 1 μM cAMP or cGMP respectively (10,000 Ci/mmol). The reaction was stopped by boiling for 90 sec. After treatment with 5′ nucleotidase for 10 min at 30°C, the resulting nucleoside was separated by chromatography over Dowex 1 × 8–400 and eluted with methanol. Each point represents the mean of two paired samples from a total of four experiments.

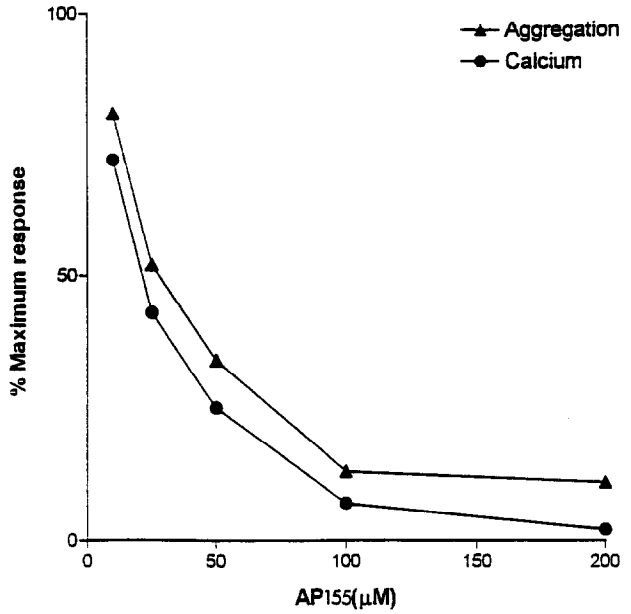


FIG. 4. Inhibition of thrombin-stimulated platelet aggregation and calcium mobilization by AP155. WP were loaded with the light sensitive Ca^{++} indicator aequorin and stimulated with 0.1 U/mL of thrombin in the presence of increasing concentrations of AP155. Aggregation was monitored by the change in light transmission and Ca^{++} mobilization by the increase in light emission using a PICA Lumiaggregometer. Each point represents the mean of two paired samples from a total of three experiments.

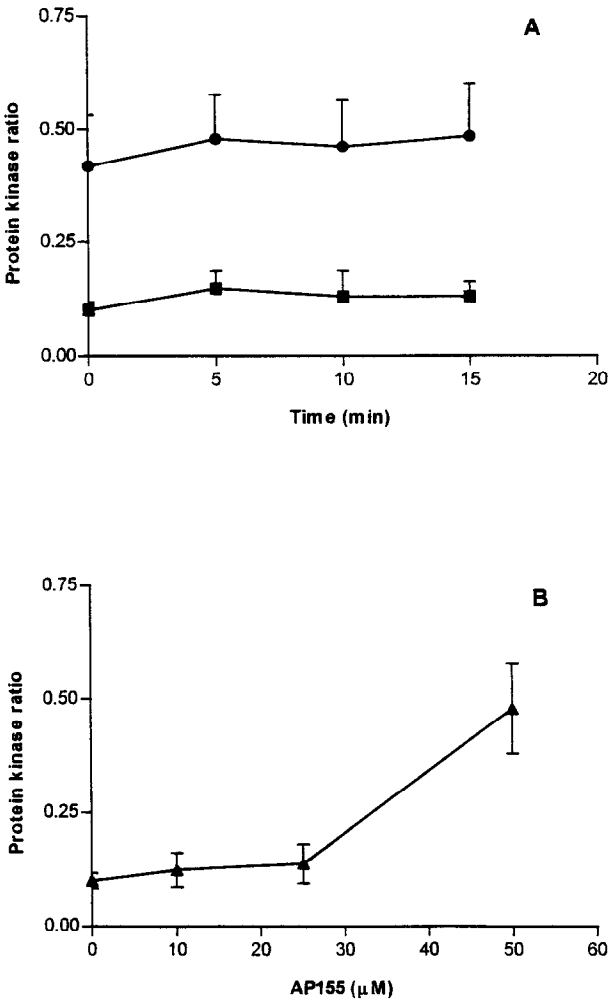


FIG. 5. Effect of AP155 on platelet cAMP-dependent protein kinase activity. Protein kinase was determined as the ratio of the phosphorylation of Kemptide substrate by platelet lysate measured without cAMP addition and to measured after excess cAMP addition. A shows the time-dependence of peptide phosphorylation in the absence (■) and in the presence (●) of 50 μM AP_{155}. B shows the effect of increasing AP155 concentrations after 5 min incubation at 37°C. Each point represents the mean ± SD of three determinations.

activation [12]. The inhibitory effects of cAMP on protein kinase C activation, Ca^{++} mobilization, fibrinogen-receptor exposure, myosin light chain phosphorylation, actin polymerization and cytoskeleton assembly are believed to be consequences of the inhibition of receptor-mediated phospholipase C activation [13].

Based on previous studies on a large number of pyrido[1,2-a]pyrimidine derivatives, we have selected the 1-piperazinyl derivative AP155, which showed the highest antiplatelet activity towards all agonists [1, 2]. This study on its mode of action has shown that AP155 can concentration dependently produce an increase in cAMP levels and can potentiate the effect of several known adenylate cyclase activators such as iloprost, PGI_2 and forskolin. Subsequently, we have demonstrated that cAMP elevation occurred via inhibition of cAMP-PDE, with a selective effect of AP155 on the cAMP high affinity soluble enzyme. Modest increases in cAMP levels, which appear to be a characteristic of low Km cAMP-PDE inhibitors, are able to produce activation of cAMP-dependent protein kinase [14, 15]. In agreement with previous studies [6], cAMP elevation by less than 50%, as generated by 50 μM AP155, produces an approximate 5-fold increase in cAMP-dependent protein kinase activity. Generally, cAMP protein kinase activating compounds have been found to be potent inhibitors of the calcium signal. It is not clear how proteins phosphorylated by cAMP protein kinase are involved in regulation of Ca^{++} mobilization [16]. Protein targets include phospholipase C isoenzymes [17] and inositol(1,4,5)P_3 receptors [18]. Nevertheless, there is considerable debate on the regulation of Ca^{++}-ATPases by cyclic nucleotides in platelets [16]. It is known that cAMP regulates the induction of fibrinogen receptor exposure [19, 20]. On the other hand, fibrinogen binding, cytosolic Ca^{++} increase and aggregability are in close correlation [21]. The data presented herein show that AP155 inhibits both platelet aggregation and cytosolic Ca^{++} increase with identical potency. Moreover, the mobilization of fibrinogen binding sites in platelets activated by ADP or thrombin is similarly blocked. In conclusion, it may be suggested that AP155 inhibits platelet function by a cAMP-mediated mechanism.

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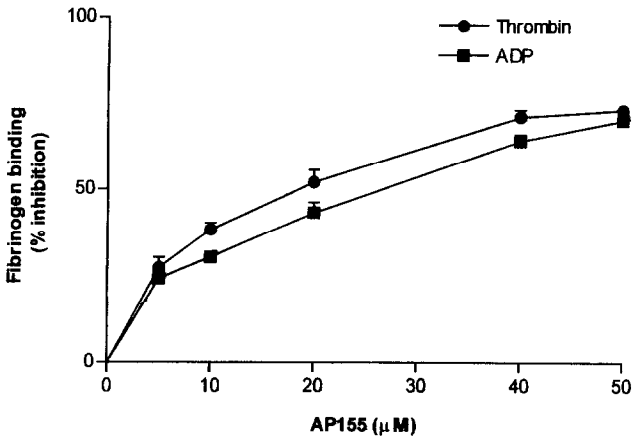

FIG. 6. Concentration-dependence of the effect of AP155 on [^{125}I]fibrinogen binding to human platelets. WP (5.0 × 10^8 plts/mL) were preincubated for 3 min with solvent or increasing concentrations of AP155, activated by 20 μM ADP (■) or 0.1 U/mL thrombin (●) for 5 min at room temperature (22°C) and finally mixed with 50 μg of [^{125}I]fibrinogen (2.0 × 10^5 cpm/μg). The stimulation of thrombin was blocked by the addition of hirudin (0.3 U/mL). Each point represents values obtained from two paired samples representative of three confirmatory experiments.

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