Decrease of platelet aggregation and spreading via inhibition of the cAMP phosphodiesterase by trapidil

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Trapidil (*N,N*-diethyl-5-methyl[1,2,4]triazolo[1,5-α]pyrimidine-7-amine) inhibits platelet spreading and aggregation induced by arachidonic acid (AA), a stable analogue of prostaglandin (PG) endoperoxides (U46619), ADP, and low concentrations of thrombin, but not by A23187 and high concentrations of thrombin. Trapidil does not affect platelet adenylate cyclase but inhibits the cAMP PDE by approx. 50%. PDE inhibition proceeds via a competitive mechanism (K_i = 0.52 mM) and is not mediated by calmodulin inhibition. Trapidil does not change the platelet basal cAMP level but potentiates an increase of cAMP induced by the stable prostacyclin analogue (6β-PGI_1). These results suggest that trapidil antiplatelet effects may be due to the inhibition of platelet PDE.

Platelet aggregation *Platelet adhesion* *Phosphodiesterase inhibitor* *cAMP* *Trapidil*

1. INTRODUCTION

An important property of cardiovascular drugs is their ability to inhibit platelet function and thus prevent the formation of intravascular occlusive and mural thrombi. It was previously demonstrated that an antianginal drug, trapidil, inhibits various platelet reactions: (i) aggregation in suspension [1,2]; (ii) spreading on a collagen-coated surface [3]; (iii) formation of surface-bound thrombi-like aggregates [3]. The mechanism of trapidil antiplatelet effects remains obscure. It was established that trapidil decreases the formation of TXA_2, a proaggregatory AA metabolite in platelets [1–3]. However, the antiplatelet properties of trapidil cannot be explained only by the inhibition of TXA_2 synthesis since: (i) trapidil completely blocks platelet aggregation and spreading induced by U46619 [1–3], a stable PG endoperoxide analogue which is not converted to TXA_2 and does not induce TXA_2 synthesis in platelets [4]; (ii) quantitative correlation between trapidil effects on AA-induced platelet aggregation/spreading and TXA_2 formation is absent [3]. A platelet cyclic nucleotide system which plays an important role in the regulation of their activity [5] may be another site of trapidil action. It was demonstrated that trapidil inhibits the activity of cAMP PDE in heart muscle [6].

Abbreviations: AA, arachidonic acid; PG, prostaglandin(s); TXA_2, thromboxane A_2; PDE, phosphodiesterase; PRP, platelet-rich plasma

2. MATERIALS AND METHODS

2.1. *Platelet isolation*

Blood was drawn from the antecubital vein of healthy donors. To obtain PRP for platelet aggregation experiments 3.8% Na citrate was used as anticoagulant (1 vol. anticoagulant : 9 vols blood); to obtain PRP for washed and gel-filtered platelet preparations acid–citrate–dextrose (1 vol. anticoagulant : 6 vols blood) [7] was used. The PRP was prepared by centrifugation of anticoagulated blood at 150 × *g* for 12.5 min, platelet-poor plasma, by repeated centrifugation (after removing PRP) at 1000 × *g* for 20 min. Washed platelets

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were isolated as in [7] using Tyrode solution (pH 6.5); gel-filtered platelets were obtained as in [8]. In both cases, platelets were finally suspended in Tyrode solution containing 2 mM Ca^{2+}, 1 mM Mg^{2+}, and 0.35% bovine serum albumin (pH 7.35).

2.2. *Platelet aggregation*

Platelet aggregation was performed using a two-channel Payton aggregometer at 37°C, a stirring rate of 900 rpm, and platelet count of 2×10^8/ml. The aggregation induced by AA, ADP and U46619 was measured in PRP; aggregation induced by A23187 and thrombin was measured in the suspension of washed platelets. The level of aggregation was determined as the maximal light transmission 3–5 min after the addition of the inducer.

2.3. *Platelet adhesion to the collagen-coated surface*

Platelet adhesion was carried out in 16.4-mm wells of culture plates (Falcon) coated with fibrillar calf skin collagen [9]. Gel-filtered platelets ($2–4 \times 10^7$) in 200 μl Tyrode solution, with or without trapidil, were introduced into each well with subsequent addition of AA, U46619 or thrombin [3,10]. The area covered with spread platelets and the number of thrombi-like aggregates on the collagen substrate were determined by scanning electron microscopy [3,10].

2.4. *Determination of platelet cAMP*

One ml washed platelets ($4–5 \times 10^8$) was placed into the aggregometer cuvette and incubated with Tyrode solution or trapidil at 37°C and a stirring rate of 900 rpm. In some experiments, 6β-PGI_1 was added to platelets 30 s after buffer or trapidil. One min after the last addition, 2 ml of 96% ethanol, preheated to 70°C, was introduced into the cuvette. After centrifugation, the supernatant was removed and the liquid phase was evaporated under vacuum. The pellet was dissolved in 0.05 M Tris–HCl, 4 mM EDTA (pH 7.5), and cAMP was determined by the high-affinity protein binding assay [11].

2.5. *Determination of platelet membrane adenylate cyclase*

Platelet membranes were isolated as in [12] as modified in [13]. To determine the activity of adenylate cyclase, 5–10 μg membrane protein were incubated for 10 min at 30°C in 50 μl medium containing 50 mM triethanolamine, 30 mM Tris–HCl, 0.3 mM EGTA, 2 mM $MgCl_2$, 1 mM 3-isobutylmethylxanthine, 1 mM dithiothreitol, 30 μM dl-propranolol, 25 μg creatine kinase (over 100 units/probe), 5 mM creatine phosphate, 50 μM ATP, 10 μM GTP, 0.1 mM cAMP, and 0.2–0.5 μCi [α-^{32}P]ATP (pH 7.4). [^{32}P]cAMP formation was determined as in [14].

2.6. *Determination of cAMP PDE activity in platelet homogenate and purified rabbit heart PDE preparation*

Platelet homogenate was obtained by freeze-thawing. PDE from rabbit heart was isolated as in [15] and calmodulin as in [16]. To determine cAMP activity, 40 μg platelet homogenate protein or 20 μg PDE preparation protein were incubated for 10 min at 30°C in 50 μl medium containing 20 mM imidazole, 5 mM $MgCl_2$, 1–10 μM cAMP and 0.2–0.3 μCi [^{3}H]cAMP (pH 7.4). In some experiments, Ca^{2+}, EGTA and calmodulin were added to the incubation medium. [^{3}H]AMP formation was determined as in [17].

3. RESULTS

Trapidil at 1 mM almost completely prevents platelet aggregation in suspension induced by AA, U46619, ADP and low concentrations of thrombin. Trapidil is less effective when more potent inducers, high thrombin concentrations and the Ca^{2+} ionophore A23187 are used (table 1). Trapidil also inhibits the reactions specific for platelet–substrate interactions: spreading of platelets and formation of thrombi-like aggregates on the sheets of spread platelets [10]. It was shown using scanning electron microscopy that trapidil prevents the spreading and formation of thrombi-like aggregates on the collagen substrate induced by AA, U46619 and low, but not high concentrations of thrombin (table 1).

Trapidil at a concentration which inhibits platelet activation in suspension and on the surface does not change the basal platelet cAMP level (fig.1A). However, trapidil potentiates the increase of cAMP induced by the stable prostacyclin analogue 6β-PGI_1 by approx. 40% (fig.1B). Trapidil does not affect the adenylate cyclase of

Table 1

Trapidil effects on platelet aggregation in suspension, platelet spreading and formation of thrombi-like aggregates on a collagen substrate

Inducer	Inhibition (%)		
	Aggregation in suspension	Thrombi-like aggregate formation	Spreading
AA (100–200 μM or 1 mM)[e]	100.0 ± 1.6[a] (3)	100 ± 0[a] (4)	96.3 ± 1.0[a] (9)
U46619 (1–2 μM)	100.0 ± 0[a] (4)	100 ± 0[b] (4)	96.9 ± 1.0[a] (4)
ADP (10 μM)	82.4 ± 1.6[b] (3)	n.d.	n.d.
Thrombin (0.1–0.2 units/ml)	89.2 ± 7.7[b] (5)	72.7[d] (1)	76.5[d] (1)
Thrombin (1–2 units/ml)	7.0 ± 4.2[d] (4)	20.2 ± 7.1[d] (2)	13.0 ± 7.6[d] (2)
A23187 (0.5–1.0 μM)	23.0 ± 5.1[c] (8)	n.d.	n.d.

Platelet aggregation in suspension was determined in an aggregometer; surface-bound thrombi-like aggregate formation and platelet spreading by scanning electron microscopy [3]. Inhibitory effects of trapidil (1 mM) were calculated with respect to the corresponding controls. The data represent the means ± SE; the number of experiments is given in brackets. Significance of the inhibitory effects was calculated using Student's paired t-test for $n > 2$. [a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$, [d] not significant. [e] Aggregation in PRP. n.d., not determined

platelet membranes. Its activity was 39.1 ± 1.8 and 38.1 ± 1.6 pmol cAMP/min per mg membrane protein in the absence and presence of 1 mM trapidil, respectively (mean ± SE, $n = 5$).

Investigation of the trapidil effect on the activity of cAMP PDE was carried out in comparison with a well-known PDE inhibitor, theophylline [18].

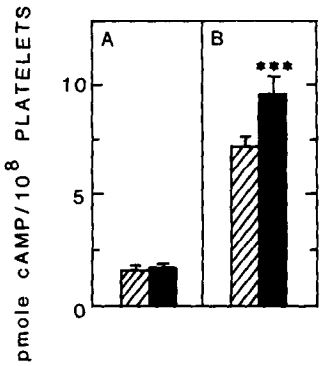

Fig.1. Effects of trapidil on the basal (A) and stimulated (by 3 mM 6β-PGI$_1$) (B) levels of platelet cAMP. Platelets were incubated in the absence (hatched bars), or presence (filled bars) of 1 mM trapidil. The data represent the means ± SE ($n = 6$). The significance of the differences between the means was determined using Student's t-test. ***$p < 0.001$.

Since both Ca^{2+}-calmodulin-dependent and -independent forms of PDE exist [19], the activity of platelet PDE was measured in the presence and absence of Ca^{2+} and calmodulin. As seen from table 2, PDE activity increases following Ca^{2+} addition and is hardly stimulated by exogenous calmodulin. These results indicate the presence of

Table 2

Effects of trapidil and theophylline on the activity of platelet cAMP PDE

Inhibitor	cAMP PDE activity			
	− Calmodulin		+ Calmodulin	
	+ EGTA	+ Ca^{2+}	+ EGTA	+ Ca^{2+}
None	269 ± 11	380 ± 10	305 ± 10	416 ± 1
Trapidil (1 mM)	165 ± 2	192 ± 12	164 ± 3	209 ± 11
Theophylline (1 mM)	143 ± 9	196 ± 14	156 ± 7	228 ± 21

cAMP PDE activity was determined at 10 μM cAMP. Additions: calmodulin (20 pM), EGTA (1 mM) and Ca^{2+} (1 mM). The data represent the means ± SE ($n = 3$). Significance of the inhibitory effects of trapidil and theophylline was calculated using Student's t-test for means. In all cases $p < 0.01$

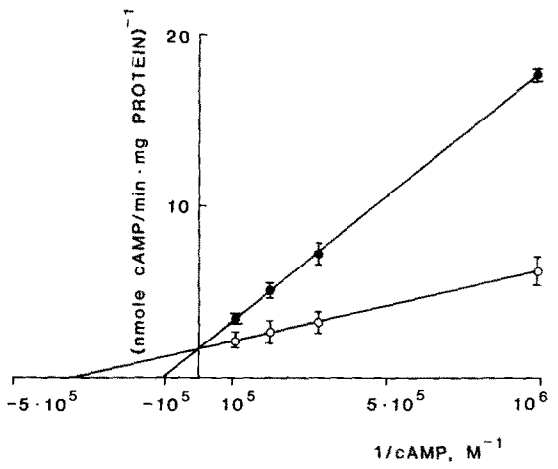

Fig.2. Inhibition of a purified PDE from rabbit heart by trapidil. The data are given in a double-reciprocal plot. Incubation was carried out without (○) or with (●) 1 mM trapidil. Means ± SE (n = 3).

endogenous calmodulin in the preparation. Trapidil inhibits PDE activity equally (by approx. 50%) in both the presence and absence of Ca^{2+} and calmodulin (table 2). Trapidil inhibits PDE as effectively as the same concentration of theophylline (table 2).

To study the mechanism of the trapidil effect on PDE, a purified preparation of PDE from rabbit heart was used. It was shown that trapidil inhibits PDE via a competitive mechanism with K_i = 5.24×10^{-4} M (fig.2). The K_i of trapidil is similar to that of theophylline which is also a competitive PDE inhibitor [18].

4. DISCUSSION

The study of trapidil effects on the platelet cAMP system presented here allows us to explain its antiplatelet action by the inhibition of PDE. It has been demonstrated that trapidil does not affect the activity of adenylate cyclase but inhibits the cAMP PDE activity of human platelets. The inhibition of PDE proceeds via a competitive mechanism which is, apparently, accounted for by the structural similarity between trapidil and the adenyl ring of cAMP. The inhibitory activity of trapidil is similar to that of theophylline. Trapidil, like other PDE inhibitors [20], does not change the basal total cAMP levels in platelets but additionally stimulates an increase in cAMP during the activation of adenylate cyclase by 6β-PGI_1. The absence of PDE inhibitor effects on the basal total cAMP level is attributed to the existence of two cAMP pools. The active pool is only a small fraction of the total one [20]. Thus, trapidil and other PDE inhibitors can prevent the activation of platelets by affecting only the functionally active pool without increasing the total cAMP level in platelets.

The previously discovered decrease of TXA_2 synthesis in platelets in the presence of trapidil [1–3] may also be explained by the influence on PDE. It was shown that a PDE inhibitor, dipyridamole, causes approximately the same decrease in TXA_2 formation in platelets as trapidil [21]. The inhibitory effect of trapidil is manifested only in whole platelets and disappears in platelet homogenates [1]. These results suggest that trapidil itself does not affect the activity of enzymes participating in AA metabolism. Probably its effect on TXA_2 synthesis is mediated by the action on the cyclic nucleotide system.

Activity of cAMP PDE in platelets is increased in the presence of Ca^{2+} and calmodulin. It is known that calmodulin regulates the activity of PDE and many other enzymes and processes in platelets [22]. Trapidil equally inhibits PDE activity in both the presence and absence of Ca^{2+} and calmodulin. Unlike specific calmodulin inhibitors [22,23] trapidil effectively prevents the activation of platelets induced only by relatively weak inducers, but not by the Ca^{2+} ionophore A23187 and high concentrations of thrombin. We also failed to demonstrate the interaction of trapidil with calmodulin (not shown). These results suggest that antiplatelet effects of trapidil are not mediated by calmodulin inhibition.

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