Biochemical Pharmacology, Vol. 44, No. 3, pp. 527–532, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

IMPORTANCE OF HEPATIC METABOLISM IN THE ANTIAGGREGATING ACTIVITY OF THE THIENOPYRIDINE CLOPIDOGREL

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(*Received* 21 *November* 1991; *accepted* 21 *April* 1992)

Abstract—The thienopyridine clopidogrel is not active *in vitro* and must be administered i.v. or orally, suggesting that metabolism is necessary for activity. To verify whether the effect after i.v. administration was consecutive to recycling by hepatic bile secretion of clopidogrel or its metabolite(s) in the digestive tract, a catheter was implanted in the choledocus of rats, preventing bile and pancreatic secretions from being excreted into the digestive tractus. Two hours after clopidogrel administration (10 mg/kg, i.v.), blood was withdrawn and platelet-rich plasma aggregation was measured after the addition of 5 μM ADP. Clopidogrel treatment was equally efficient for sham-operated and catheterized animals (% inhibition of platelet aggregation: 76% and 59%, respectively) suggesting that the i.v. effect of clopidogrel was independent of re-absorption of biliary-excreted products and consequently that enteric metabolism is not necessary for activity. The antiaggregating activity of clopidogrel in rats before and after functional hepatectomy by a porto-jugular shunt was then studied. A great difference between treated animals was observed 30 min after i.v. administration of 25 mg/kg of clopidogrel. Per cent inhibition of platelet aggregation was 76% and 6% ($P < 0.001$) for sham-operated and hepatectomized animals, respectively. Similar results were obtained after intraduodenal administration of clopidogrel, showing that the treatment was completely ineffective in hepatectomized animals. In isolated, blood-perfused rat livers, clopidogrel inhibited ADP-induced platelet aggregation, thereby supporting the theory that the activity of clopidogrel is highly dependent on hepatic metabolism.

Ticlopidine [1–3] and its potent analogue clopidogrel [4] are powerful inhibitors of ADP-induced platelet aggregation. They also inhibit platelet aggregation induced by low concentrations of other agonists, apparently by blocking the amplification of platelet activation by released ADP [5]. At present, the molecular mechanism which explains the anti-ADP selectivity of the thienopyridines has not yet been found. The thienopyridines are not active *in vitro* on ADP-induced platelet aggregation, and it is now well established that these compounds must be administered orally or, for clopidogrel only, by the i.v. route to be active. However, the way in which thienopyridines are metabolized endogenously into possible active compound(s) is still not known. It has been suggested that the active metabolite of ticlopidine could be produced in small quantities at the intestinal level after oral administration [6] and could affect platelets only at the mesenteric and portal levels. This hypothetical metabolite must be biologically unstable because the antiplatelet activity was not transferred in cell-free plasma from treated animals to platelets from control subjects. The inhibitory effect of the thienopyridines on platelet functions is permanent, since it persists several days after withdrawal of the drug and disappears in proportion to platelet renewal (P. Savi, unpublished observations).

The aim of our study was therefore to examine the relative importance of intestinal and/or hepatic metabolisms in the appearance of the antiaggregating effect of clopidogrel.

MATERIALS AND METHODS

Chemicals. ADP was purchased from Boehringer Mannheim (Mannheim, F.R.G.). Collagen was from the Sigma Chemical Co. (L'Isle d'Abeau, France). Thrombin (71 NIH U/mg) was from Hoffman La Roche (Basel, Switzerland). Clopidogrel (*d*-methyl(2- chlorophenyl)- 5- (4,5,6,7- tetrahydrothieno) (3,2-*c*)pyridinyl)acetate, hydrogensulfate) (Fig. 1) and the corresponding inactive levorotatory enantiomer (SR 25989) were synthesized by Sanofi Recherche (Toulouse, France).

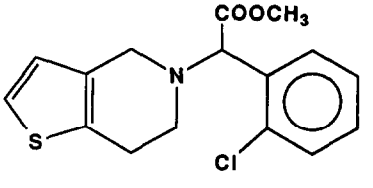

Fig. 1. Structure of clopidogrel.

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† Abbreviations: PRP, platelet-rich plasma; PG(E_1,I_2,D_2), prostaglandin(E_1,I_2,D_2).

Animals. All the experiments were performed on female Sprague–Dawley rats (CD-COBS), weighing between 250 and 300 g, provided by Charles River (Saint Aubin les Elbeuf, France). Animals were housed in groups of 10 and were starved 16 hr before the experiment.

Determination of the antiaggregating activity of clopidogrel ex vivo. Animals were treated with clopidogrel or SR 25989 at the indicated doses or with vehicle, either orally by gavage in a 5% gum arabic solution (10 mL/kg) or intravenously in saline (1 mL/kg) through a jugular vein. Blood samples of ether-anaesthetized rats were collected into a 3.8% trisodium citrate solution (9:1 v/v) at various time periods after administration, by puncture of the abdominal aorta. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood (500 *g*, 10 min, 15°). Platelet-poor plasma was obtained by further centrifugation of the pellet (3000 *g*, 15 min) and used to dilute the PRP in order to adjust the platelet count to 10^6 cells/μL in the final suspension. Platelet aggregation was monitored by the turbidimetric method of Born [7] on a dual-channel Chrono-Log aggregometer. PRP was equilibrated at 37° for 1 min under constant stirring (900 rpm) and aggregation was induced by 2.5 μM ADP or by the other agonists at the indicated concentrations. The extent of aggregation was estimated quantitatively by measuring the maximum curve height above baseline level.

Antiaggregating activity of clopidogrel in cholecdochostomized rats. Animals were anaesthetized by intraperitoneal injection of nembutal (40 mg/kg, i.p.) and the liver exposed with a transverse incision of the abdominal wall. The bile duct was cannulated with a polyethylene catheter (i.d. 0.58 mm, o.d. 0.96 mm; Biotrol Pharma, France) according to the method of Waynforth [8]. Rats were sham-operated on in the same way except that they were not implanted with the catheter. Clopidogrel (10 mg/kg, i.v.) was administered intravenously through a jugular vein 10 min later. Control rats received only the vehicle (saline). Blood was withdrawn 2 hr later and aggregation of the PRP was induced by 5 μM ADP as indicated previously.

Antiaggregant activity of clopidogrel in hepatectomized rats. Rats were anaesthetized by intraperitoneal injection of nembutal (40 mg/kg, i.p.) and the jugular and portal veins were exposed. A polyethylene catheter (i.d. 1.19 mm, o.d. 1.70 mm; Biotrol Pharma, France) was inserted between the portal and the jugular veins, according to the method of Poser and Jahws [9]. This functional shunt allowed all the mesenteric blood flow to be diverted from the liver into the main circulation. Rats were sham-operated similarly except that no cathether was inserted. Clopidogrel was then administered (25 mg/kg) either intravenously in saline or intraduodenally in water. Controls received only the vehicles. Blood was withdrawn 30 min later and ADP-induced aggregation of the PRP was measured as indicated previously.

Determination of the antiaggregating activity of clopidogrel in a perfused liver in situ. Liver perfusion was performed *in situ* according to the method of Miller [10]. Rats were anaesthetized by an intraperitoneal injection of nembutal (40 mg/kg, i.p.), the liver was exposed and the bile duct was cannulated with a polyethylene catheter (i.d. 0.58 mm, o.d. 0.96 mm; Biotrol Pharma, France). The portal vein was then cannulated with an 18-gauge needle and perfused immediately with an oxygenated Ringer buffer (NaCl 154 mM, KCl 5.6 mM, $CaCl_2$ 2.2 mM, $NaHCO_3$ 6 mM, glucose 1 g/L, pH 7.4) containing 10 IU/mL of heparin. The perfusate flow was maintained at a constant rate of approximately 7 mL/min using a peristaltic pump (Gilson, Villiers le Bel, France). A second cannula was inserted into the lower part of the vena cava and the upper part was ligatured, allowing the perfusate to flow out. The liver was then transferred with the animal into a 37°-thermo-controlled room (Gilson) and the perfusion was continued in order to eliminate all the blood contained in the liver. The cannulae were then connected to a perfusion apparatus with a recirculating perfusate made of fresh anticoagulated rat blood diluted in Ringer buffer (3:1 v/v) containing heparin (10 IU/mL final). The total vlume of the perfusate was 40 mL and was oxygenated with a stream of 95% O_2, 5% CO_2 (6 L/min), at a negative pressure of 1 cm of water. The perfusion was performed at a constant pressure of 15 cm of perfusate. Clopidogrel or SR 25989 (1 mM final) in solution in saline was added to the perfusate 15 min after the beginning of the perfusion. Control experiments were made by adding only the vehicle. After 45 min, 5 mL of blood were withdrawn from the perfusion apparatus, mixed in a trisodium citrate (93 mM)–citric acid (7 mM)–dextrose (140 mM) solution (9:1 v/v), and PRP was obtained by centrifugation (350 *g*, 10 min, 15°). The PRP was acidified with trisodium citrate–citric acid–dextrose solution (2:1 v/v) and centrifuged (700 *g*, 10 min, 15°). The platelet pellet was resuspended in platelet-poor plasma from untreated animals and the platelet count was adjusted to 10^6 cells/μL. Platelet aggregation was measured after the addition of ADP (5 μM) as indicated previously.

Statistical analysis. The results are expressed as means ± S.D. Statistical analysis was performed by Kruskal–Wallis non-parametric analysis of variance taking $P < 0.05$ to indicate a significant difference.

RESULTS

Determination of the antiaggregating activity of clopidogrel

As shown in Fig. 2, ADP-induced platelet aggregation was inhibited *ex vivo* after a single i.v. administration of clopidogrel (10 mg/kg, i.v.). When given p.o. at the same dose, clopidogrel was absorbed rapidly as measured by the onset of its antiaggregating activity, which closely parallelled that measured after i.v. administration. The maximum antiaggregating effect was obtained 2 hr after administration and stayed at the same level for 1–2 days (not shown). The inhibitory activity declined thereafter but remained significant for at least 5 days. As already shown, the antiaggregating effect of clopidogrel was directed mainly against ADP, even at high agonist concentrations (Table 1). Two hours after oral administration (10 mg/kg), platelet aggregation

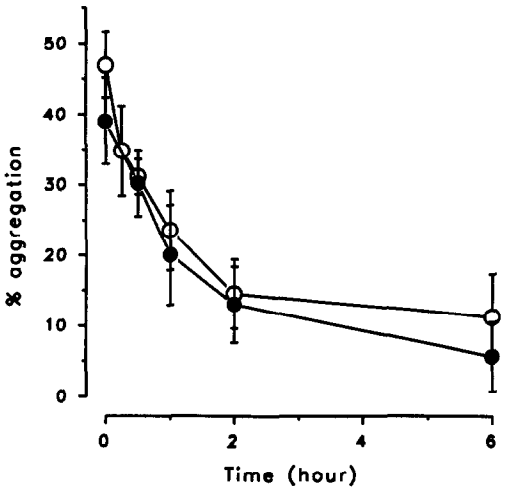

Fig. 2. Time course of the antiaggregant activity of clopidogrel *ex vivo*. Clopidogrel (10 mg/kg) was administered p.o. (●) or i.v. (○). At various time intervals, platelet aggregation was induced *ex vivo* by 2.5 μM ADP. Each point is the mean ± SD (N = 6).

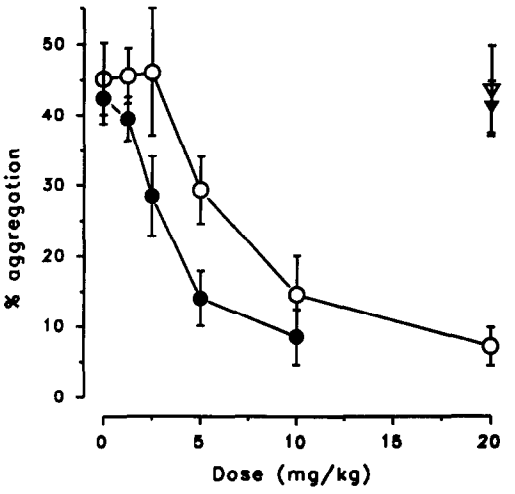

Fig. 3. Effect of clopidogrel on ADP-induced platelet aggregation *ex vivo*. Clopidogrel (circles) or SR 25989 (triangles) was administered p.o. (filled symbols) or i.v. (empty symbols) 2 hr before induction of platelet aggregation by 2.5 μM ADP. Each point is the mean ± SE (N = 6).

Table 1. Effect of clopidogrel on platelet aggregation induced by various agonists

	ADP (μM)		Collagen (μg/mL)		Thrombin (IU/mL)	
Concentration	0.5	10	1	10	0.1	1
% Inhibition	100	100	90	90	96	5

Clopidogrel was administered to female rats p.o. (10 mg/kg). Aggregation of the PRP was induced 2 hr later by various agonists at the indicated concentrations.

Results shown are the means of at least three independent experiments.

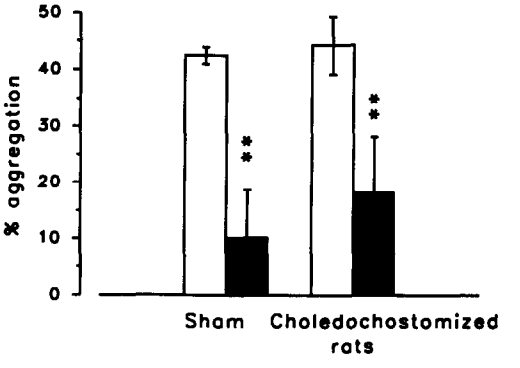

Fig. 4. *Ex vivo* antiaggregant activity of clopidogrel on platelets from choledochostomized or sham-operated rats. Clopidogrel was administered i.v. (10 mg/kg) (filled bars) to sham-operated or choledochostomized rats. Vehicle was administered to controls (empty bars). Platelet aggregation was induced *ex vivo* with ADP (5 μM) 2 hr later. Each point is the mean ± SD (N = 5). Statistical analysis: $^{**}P < 0.01$ as compared with the vehicle-treated group (Kruskal–Wallis test).

induced by low concentrations of collagen (1 μg/mL) or thrombin (0.1 IU/mL) was also inhibited by clopidogrel (Table 1). This effect could be overcome by higher thrombin concentrations, but the aggregating effect of collagen remained totally blocked. Such an effect has already been seen with ticlopidine [6].

Dose–response studies showed that clopidogrel inhibited ADP-induced platelet aggregation *ex vivo* in a dose-dependent manner (Fig. 3). Nearly maximal inhibition of ADP-induced platelet aggregation was achieved 2 hr after oral administration of 5 mg/kg or i.v. injection of 10 mg/kg of clopidogrel. From these dose–response curves, ID_{50} values (doses which inhibit 50% of the ADP-induced platelet aggregation) were 3.5 and 5 mg/kg after oral and i.v. administration, respectively. Under the same experimental conditions, i.v. or oral administration of the levorotatory enantiomer of clopidogrel (SR 25989) did not significantly affect ADP-induced platelet aggregation, confirming results shown previously [11].

Antiaggregating activity of clopidogrel in choledochostomized rats

A catheter was implanted in the choledocus of female rats, preventing bile and pancreatic secretions from being excreted. Under these experimental conditions, no differences were shown between controls with or without the catheter [% aggregation: 42.4 ± 0.04 (N = 5) vs 44.2 ± 2.26 (N = 5), respectively] (Fig. 4). When given i.v., a 2-hr pretreatment of clopidogrel (10 mg/kg) showed a similar inhibition

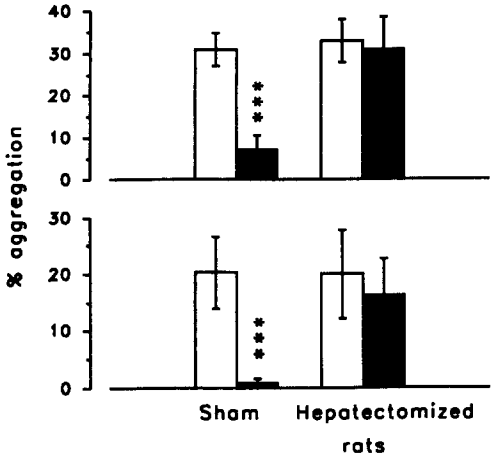

Fig. 5. *Ex vivo* antiaggregant activity of clopidogrel on platelets from hepatectomized or sham-operated rats. Vehicle (empty bars) or clopidogrel (25 mg/kg) (filled bars) was administered i.v. (upper panel) or i.d. (lower panel) to sham-operated or hepatectomized rats. Platelet aggregation was induced *ex vivo* with ADP (5 μM) 30 min later. Each point is the mean ± SD of at least five animals. Statistical analysis: ***$P < 0.001$ as compared with the vehicle-treated group (Kruskal–Wallis test).

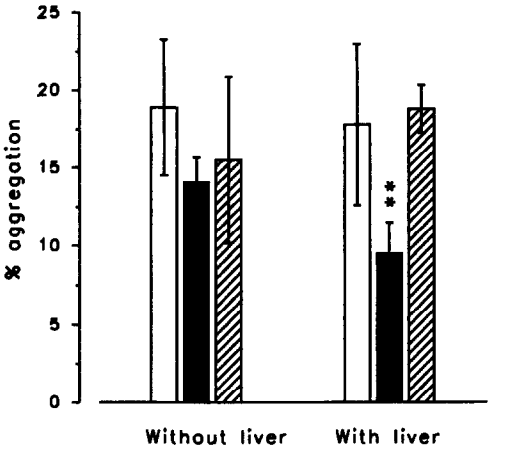

Fig. 6. Antiaggregant activity of clopidogrel and SR 25989 after perfusion in an isolated liver. Clopidogrel (filled bars), SR 25989 (shaded bars) (1 mM) or the vehicle (empty bars) was perfused in isolated rat livers as indicated in Materials and Methods. The compounds were also perfused under the same experimental conditions without the organ. ADP-induced platelet aggregation was measured 45 min later. Each point is the mean ± SD of four animals. Statistical analysis: **$P < 0.001$ as compared with the vehicle-treated group (Kruskal–Wallis test).

of ADP-induced aggregation of platelets isolated from either sham-operated or choledochostomized rats (76% and 59% inhibition of ADP-induced platelet aggregation respectively). No statistical difference was observed between the effect of clopidogrel in sham-treated and choledochostomized rats ($P > 0.05$).

Antiaggregating activity of clopidogrel in hepatectomized rats

The implication of the hepatic metabolism of clopidogrel was evaluated in female rats before and after insertion of a porto-jugular shunt leading to a functional hepatectomy. Thirty minutes after i.v. administration of clopidogrel (25 mg/kg, i.v.) or vehicle, no differences were observed between the controls: per cent aggregation was 30.9 ± 1.7 ($N = 6$) and 32.9 ± 2.3 ($N = 7$) for sham-operated and hepatectomized animals, respectively (Fig. 5). In contrast, a great difference was observed between the treated animal suggesting that the presence of the liver was necessary for the appearance of the antiaggregating activity of clopidogrel (76% and 6% inhibition of platelet aggregation for sham-operated and hepatectomized rats, respectively). Figure 5 indicates that similar results were obtained after i.d. administration of 25 mg/kg of clopidogrel.

Antiaggregating activity of clopidogrel after perfusion in an isolated liver

In order to ascertain whether hepatic metabolism is necessary for the appearance of the antiaggregating activity of thienopyridines, heparinized blood was recirculated in the presence of clopidogrel in an isolated, perfused rat liver. Biliary secretion, glucose concentration of the perfusate, pH of the perfusate and the physical aspect of the liver were criteria for the validity of each experiment. During the experiment, the secretion of bile was not less than 50 μL/hr, in agreement with Graf *et al.* [12]. Glucose concentration as measured by BM-Test-Glycemie (Boehringer Mannheim, F.R.G.), was tripled during the 1-hr perfusion. This result corresponded to that already described by Bartosek *et al.* [13]. A slight decrease in pH (0.2 U) was noticed during the first 30 min as described by Miller and Griffin [14]. Addition of clopidogrel or SR 25989 (1 mM) to the perfusion system was without significant influence on these parameters. After a 45-min recirculation period, clopidogrel significantly diminished (50% inhibition, $P < 0.01$, $N = 4$) ADP-induced aggregation of rat platelets whereas SR 25989 (1 mM) was without effect (Fig. 6). Under the same experimental conditions, but omitting the liver, neither clopidogrel nor SR 25989 showed any effect on ADP-induced platelet aggregation.

DISCUSSION

The antiplatelet activity of ticlopidine and its more potent analogue clopidogrel resides in their specific inhibitory effect on exogenous as well as on released ADP. To elucidate the mode of action of these compounds, it is worthwhile knowing which activation process in ADP-stimulated platelets is modified by these compounds. It has been shown that ticlopidine inhibits the binding of fibrinogen to its platelet receptor, the glycoprotein GP IIb–IIIa complex [15, 16], this is probably the consequence of an earlier impact as it has been demonstrated recently that PCR 4099 (the racemic form of

clopidogrel) neither quantitatively nor qualitatively modified the GP IIb–IIIa complex [17]. However, since the exact site of action of the thienopyridines is not yet elucidated, it is now evident that these compounds have no effect on cyclooxygenase, unlike aspirin or sulfinpyrazole, or on thromboxane synthetase, unlike imidazole derivatives. They do not inhibit the uptake of adenosine or cAMP phosphodiesterase activity, both of which actions are exhibited by dipyridamole, and do not stimulate adenylate cyclase as do PGE_1, PGI_2 and PGD_2 [11]. However, ticlopidine and clopidogrel were found recently to neutralize selectively the ADP inhibition of PGE_2-activated platelet adenylate cyclase in rats and rabbits [11].

Ticlopidine and clopidogrel have no effect *in vitro* on ADP-induced platelet aggregation and must undergo metabolism to exhibit irreversible antiplatelet activity. Nevertheless, the putative active metabolite(s) is not present in the plasma, urine or bile secretions, since no antiaggregating activity was found in these biological fluids after ticlopidine or clopidogrel administration (P. Savi, unpublished observations). Up to now, the organ responsible for the metabolism of thienopyridines has not been shown but, because ticlopidine is only active after oral administration, the hypothesis of an intestinally generated metabolite has emerged [6]. One of the greatest differences between ticlopidine and clopidogrel, in addition to the greater antiaggregating activity of the latter, is the fact that clopidogrel is active after i.v. administration. This fact allowed us to determine the kinetics of its antiaggregating effect, together with the dose–response relationships after both i.v. and oral administration. The results indicate equivalent bioavailability of the two routes, as indicated by the high level of similarity between the areas under the curves. Furthermore, our studies confirm the stereoselectivity of clopidogrel, since the administration of a high dose of the levorotatory enantiomer (SR 25989) was without effect on ADP-induced platelet aggregation.

Having improved the experimental conditions for the detection of an optimal effect of clopidogrel, the relative importance of hepatic metabolism for the antiplatelet effect was assessed. In order to avoid any involvement of the digestive tract in the activation process, clopidogrel was administered i.v. It was verified first whether the appearance of the antiaggregating activity was consecutive to recycling by hepatic secretions of clopidogrel itself or its metabolites into the digestive tract, as has been shown previously for i.v. treatment with indomethacin [18]. When a catheter was implanted in the choledocus, the *ex vivo* antiaggregating activity of clopidogrel was not modified. This observation indicates that the i.v. effect of clopidogrel is independent of reabsorption of biliary-secreted products and supports the hypothesis that the activating metabolism of thienopyridines does not occur at the intestinal level.

After implantation of a porto-jugular shunt diverting the portal blood flow to the jugular vein, resulting in functional hepatectomy, clopidogrel lost all of its antiaggregating activity after intravenous or intraduodenal administration. This observation demonstrated therefore that the liver is essential for the activating metabolism of clopidogrel. A similar model was used to determine the selectivity of oral aspirin as an inhibitor of platelet and vascular cyclooxygenase activity in rats [19], showing that deacetylation of aspirin could be reduced by the implantation of a porto-jugular shunt. The preponderant role of the liver was further ascertained by using an isolated, perfused rat liver system where clopidogrel, but not its levorotatory enantiomer SR 25989, displayed an activity against ADP-induced platelet aggregation.

In conclusion, we have demonstrated that the ADP-selective antiaggregating effect of clopidogrel is generated in the liver by an unknown activating metabolism of the native drug. Intestinal metabolism or hepatic recycling seems not to be involved. The newly generated compound(s), of a transient nature, act by inhibiting a step in signal transduction between the ADP receptor and the activation of fibrinogen binding sites. The actual site of action of clopidogrel and ticlopidine remains elusive. Further studies may increase our knowledge of the stimulus–response coupling mechanisms involved in platelet activation by ADP.

Acknowledgements—The authors wish to thank A. J. Patacchini and P. E. Keane for their helpful comments on the manuscript.

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