British Journal of Pharmacology (1997) 122, 682–686

Effects of dopamine and selective dopamine agonists upon platelet accumulation in the cerebral and pulmonary vasculature of the rabbit

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1 A selection of novel compounds were shown to exhibit dopaminergic activity *in vitro*.

2 ^{111}Indium-labelled platelets were continuously monitored in the cerebral and pulmonary vasculature of anaesthetized rabbits. The effects of dopamine and selective dopamine receptor agonists on ADP and thrombin induced platelet accumulation were recorded.

3 Pretreatment with dopamine (2 mg kg^{-1} min^{-1}, i.v.) significantly reduced ADP (20 μg kg^{-1}, i.v.) induced platelet accumulation in the pulmonary vasculature whereas lower doses had no effect.

4 Dopamine (100 μg kg^{-1} min^{-1} intra-carotid, i.c.) potentiated thrombin (90 u kg^{-1}, i.c.) induced platelet accumulation in the cerebral vasculature whereas higher doses (1–2 mg kg^{-1} min^{-1}) inhibited accumulation.

5 The selective dopamine receptor agonists tested did not significantly inhibit platelet accumulation induced by ADP or thrombin. Two of these selective agonists, at doses higher than the intended therapeutic doses, induced thrombocytopaenia and an associated increase in platelet accumulation in the lung in response to thrombin.

6 These results extend previous *in vitro* studies regarding the dual actions of dopamine upon platelets and show for the first time the effects of selective dopamine receptor agonists upon platelet aggregation *in vivo*.

Keywords: Platelet aggregation; thrombosis; thrombin-coagulation; dopamine

Introduction

Dopamine stimulates both α- and β-adrenoceptors as well as specific dopamine receptors. It selectively dilates renal and mesenteric arterioles, increases the inotropic and chronotropic actions of the heart and stimulates mild vasoconstriction (Goldberg, 1972). It has been used clinically in the treatment of congestive heart failure, myocardial dysfunction, shock and Parkinson's disease (Goldberg, 1972; Tarazi, 1974). At high doses, dopamine causes vasoconstriction and impaired renal function (Lorenz *et al*., 1995). Specific dopamine receptor agonists have been shown to elicit the beneficial effects of vasodilatation, lowered blood pressure and increased kidney perfusion (Frederickson *et al*., 1985; Hughes *et al*., 1986; Elliott *et al*., 1990) without the side-effects associated with dopamine (Shusterman, 1993).

Human platelets take up dopamine by a specific, temperature and energy dependent process (Dean & Copolov, 1989), which is not influenced by dopamine D_1 or D_2 receptors (Dean *et al*., 1992). Furthermore, 2×10^{-4} M dopamine causes shape changes in rabbit platelets *in vitro* but not aggregation and 2×10^{-6} M dopamine potentiates adenosine 5′-diphosphate (ADP)-induced aggregation (Ahtee & Michal, 1972). This *in vitro* potentiation is blocked by phentolamine, indicating the involvement of α-adrenoceptors. Higher concentrations of dopamine have been shown to inhibit ADP-, adrenaline- and collagen-induced aggregation *in vitro* (Braunstein *et al*., 1977). Moreover, inhibition of adrenaline-induced aggregation was not prevented by propranolol and these inhibitory effects have been suggested to occur via D_1-like receptors (De Keyser *et al*., 1988). Stimulation of these D_1-like receptors may therefore have potentially useful clinical applications. However, the behaviour of platelets *in vitro* is poorly predictive of platelet function *in vivo*. We have, therefore, developed an animal model of thromboembolism (Page *et al*., 1982; May *et al*., 1990) in which ^{111}In-labelled platelets are continuously monitored in the cerebral and pulmonary regions, allowing accumulation responses to a variety of platelet agonists to be measured *in vivo*. This technique has previously been employed to determine the effects of a number of anti-thrombotic drugs (Thiemermann *et al*., 1990; Paul *et al*., 1993; Liu *et al*., 1994), profibrinolytics (May *et al*., 1992; Paul *et al*., 1993) and endogenous factors such as nitric oxide (May *et al*., 1991) upon platelet function *in vivo*.

In the present experiments, the effects of dopamine and selective dopamine agonists upon ADP and thrombin induced accumulation of ^{111}Indium-labelled platelets in the pulmonary and cerebral vasculature of the rabbit have been investigated.

Methods

In vitro *pharmacology of novel dopaminergic compounds*

D_1-like activity: superfused rabbit splenic artery Male, New Zealand White rabbits were killed and the splenic artery isolated and cut into 2–3 mm long rings. These preparations were mounted in a superfusion set at a resting tension of 2 g and continuously washed with modified Krebs solution at a rate of 2 ml min^{-1}. The superfusion solution was gassed (95% O_2 and 5% CO_2), heated (37°C) and contained propranolol (1 μM), indomethacin (5 μM) and EDTA (10 μM). Rings were α-blocked by a 30 min incubation with phenoxybenzamine (3 μM) and continuously contracted with the thromboxane A_2-mimetic U46619 (9,11-dideoxy-9α,11α-epoxy-methanprostaglandin $F_{2\alpha}$, 0.03 μM) in the presence of IBMX (isobutylmethylxanthine, 3 μM).

Dopamine and dopamine-receptor agonists or antagonists were then cumulatively administered, with a 30 min interval

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between dose-response curves, and their relaxant effects evaluated in terms of pD_2 ($-\log EC_{50}$) values.

D_2-like activity: superfused rabbit ear artery Rings of rabbit central ear artery (2–3 mm long) were mounted in a perspex Mayflower chamber under a resting tension of 1 g and continuously washed at 2 ml min^{-1} with modified Krebs solution. The superfusion solution was gassed (95% O_2 and 5% CO_2), heated (35°C) and contained EDTA (10 μM), desipramine (0.1 μM) and corticosterone (30 μM). The preparations were electrically stimulated with field square wave pulses every 5 min (10 Hz, 1 ms, 50–100 mA, 500 ms duration) and equilibrated for 1 h. Dopamine and dopamine receptor agonists were cumulatively administered with a 30 min interval between curves and their inhibitory effect on the electrically-induced contraction expressed as pD_2 values.

α_1-Adrenoceptor activity: rabbit aorta Rings of rabbit aorta, 3 mm long, were mounted in 25 ml organ baths containing modified Krebs solution at a resting tension of 5 g. The solution was gassed (95% O_2 and 5% CO_2), heated (37°C) and contained EDTA (10 μM), desipramine (0.1 μM), corticosterone (30 μM) and propranolol (3 μM). Cumulative concentration-response curves for dopamine or dopamine receptor agonists were obtained before and after 60 min incubation with prazosin (10 nM).

a_2-Adrenoceptor activity: guinea-pig right atrium Male Hartley guinea-pigs (Bettinardi, Novara, Italy) weighing 0.4–0.5 kg were killed by cervical dislocation and the heart rapidly removed. The right atrium was mounted in a 25 ml organ bath containing modified Krebs solution at a resting tension of 1 g. The bathing solution was gassed as before, heated (34°C) and contained 10 μM EDTA. After incubation for 30 min with desipramine (0.1 μM) and atropine (1 μM), the preparations were electrically stimulated with field pulses every 6 min (1 Hz, 0.5 ms, 30–40 V, 10 s duration). Dopamine or dopamine receptor agonists were cumulatively added and their inhibitory effects on the tachycardic response to electrical stimulation before and after yohimbine (0.3 μM) expressed as pD_2 values.

In vivo *methodology*

Animals The study was carried out on New Zealand White, male rabbits weighing 2.0–3.0 kg (Froxfield, Farms, Petersfield, Hampshire U.K.). Animals were fed a normal diet and received water *ad libitum*.

^{111}In labelling of platelets Full details of the protocol for the isolation and radiolabelling of rabbit platelets have been described elsewhere (May *et al*., 1990). Briefly, 9 ml blood was collected from the right ear artery into 3.8% (w/v) trisodium citrate and centrifuged (225 $g \times 15$ min) to obtain platelet rich plasma (PRP). PRP was buffered in Ca^{2+}-free Tyrode solution containing prostaglandin E_1 (300 ng ml^{-1}) (CFTP) and centrifuged at 675 $g \times 15$ min. After removal of the supernatant, the surface of the platelet pellet was washed with CFTP. The platelets were gently resuspended in 1 ml of CFTP and incubated for 2 min at 37°C with 1.8 MBq ^{111}Indium oxine. After a further centrifugation (640 $g \times 15$ min) the supernatant containing free ^{111}In oxine (approximately 0.7 MBq) was removed and the platelets resuspended in 2 ml CFTP.

Experimental procedure Neurolept analgesia was induced in rabbits with diazepam (4 mg kg^{-1}, i.p.) followed 10 min later by Hypnorm (0.4 ml kg^{-1}, i.m.). Analgesia was maintained by i.m. injections of Hypnorm (0.1–0.2 ml kg^{-1}) as necessary (approximately 30 min intervals). The left common carotid artery was cannulated in the direction of blood flow for intracarotid (i.c.) bolus injections and infusions. Intravenous bolus injections and infusions were made via a butterfly cannula placed in a left marginal ear vein. Agonists were flushed into the circulation with 0.5 ml 0.9% saline. ^{111}In-labelled platelets were administered via a left marginal ear vein and allowed to equilibrate in the circulation for 40 min before challenge with platelet agonists. Mean baseline values were calculated from 30 readings taken in the 30 s immediately preceding each experimental recording period. All animals were given an initial dose of ADP (100 μg kg^{-1}, i.c. or 10 μg kg^{-1}, i.v.) to check the viability of radiolabelled platelets (May *et al*., 1990).

Circulating ^{111}In-labelled platelets were continuously monitored in the pulmonary and cerebral circulations with 2.5 cm crystal scintillation probes placed over the thorax and against the head. Counts were estimated with a dual-channel gamma spectrometer (Nuclear Enterprises NE461) and logged with the aid of a special application interface (AIMS 8000, Mumed Ltd.) by a microcomputer (Dell System 200).

Reagents and drugs

ADP, atropine, bovine plasma thrombin, citric acid, corticosterone, desipramine, dopamine, IBMX, indomethacin, prazosin, propranolol, prostaglandin E_1, trisodium citrate, U-46619 and yohimbine were obtained from Sigma. Ca^{2+}-free Tyrode solution from Gibco, diazepam (Valium) from Roche, EDTA from Carlo Erba, Hypnorm (fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}) from Janssen Animal Health, ^{111}Indium oxine from Amersham International and phenoxybenzamine from RBI.

The following drugs were synthesized by Zambon Group S.p.A. (Bresso, Italy): Z1046 ((**S**)-6-[[6-[[2-(2-methoxyphenoxy)ethyl]amino]hexyl]propylamino]-5,6,7,8-tetrahydro-1,2-naphtalenediol dihydrochloride), Z10997A ((**S**)-N-propyl-N-[6-[2-(3,5-dihydroxyphenyl) ethyl amino)hexyl]-5,6-dihydroxy-1,2,3,4-tetrahydro-2-napthylamine) and Z11410 ((**S**)-N-propyl-N-(6-(2-(2-oxo-3H-1,3-benzothiazol-6-yl)ehylamino)hexyl)-5,6-dihydroxy-1,2,3,4-tetrahydro-2-naphthylamine dihydrobromide); (D_1 and D_2-like agonists); fenoldopam mesylate (FDPM) (a D_1-like selective agonist); dipropyldopamine (DPDA, a D_2-like selective agonist).

Drugs were dissolved in saline and infused for 40 min, i.c., when tested against thrombin, or i.v. (via a left marginal ear vein) when tested against ADP. Some of the drugs tested produced small changes in baseline counts (see Tables 2 and 3) in which case the final data were adjusted accordingly. Infusions were continued for the duration of the recording period.

Statistics

In vitro results are expressed as pD_2. All *in vivo* values are expressed as means ± s.e.mean ($n = 5$). Responses to platelet agonists are expressed as maximum percentage increase in counts above stable baseline values before injection of the agonist (max % increase). Control and experimental responses to ADP were compared by a paired *t* test; one-way ANOVA followed by a multiple comparison test (Tukey test) was used to compare all other *in vivo* data.

Results

In vitro *studies*

These results are shown in Table 1. The agonists Z10997A and Z11410 were more potent D_1 (and D_2) agonists than dopamine as measured *in vitro* by vascular relaxation of precontracted (thromboxane A_2 (TxA_2) mimetic) rabbit splenic artery. Dipropyldopamine (DPDA) was a less potent D_1 agonist than dopamine, and its D_2 activity was comparable to that of dopamine, as measured by inhibition of neurogenic contraction evoked by electrical stimulation in the rabbit ear artery. Fenoldopam (FDPM) was a pure D_1-agonist, having no D_2 activity.

All of the drugs tested also showed α-adrenoceptor activity (contraction of rabbit aorta, α_1 and inhibition of tachycardic effect in guinea-pig-atrium, α_2) to varying degrees; see Table 1.

In vivo *studies*

Pretreatment with dopamine (30–100 μg kg^{-1} min^{-1}, i.v.) had no significant effect ($P>0.05$) on subsequent ADP (20 μg kg^{-1})-induced platelet accumulation in the pulmonary vasculature (Table 2). However, a higher dose of dopamine (2 mg kg^{-1} min^{-1}) significantly ($P<0.05$) inhibited-ADP induced platelet accumulation in this vascular bed.

Dopamine infusion (100 μg kg^{-1} min^{-1}, i.c.) significantly ($P<0.05$) potentiated the maximum % increase in platelet counts in the head following administration of thrombin (90 u kg^{-1}, i.c.) from 95.3±4.8 with saline to 140.7±13.8 (Figure 1a). Doses lower than 100 μg kg^{-1} min^{-1} had no effect on platelet accumulation (Table 3). Once again, a higher dose of dopamine reduced platelet accumulation in the head, from 111.4±7.3 (saline control) to 77.9±13.6 (1 mg kg^{-1} min^{-1}; not significant, $P>0.05$) and to 59.4±6.5 (2 mg kg^{-1} min^{-1}; significant, $P<0.05$), (Figure 1b).

ADP-induced platelet accumulation in the pulmonary vasculature was not significantly ($P>0.05$) affected by infusion of the specific dopamine agonists tested (see Table 2). None of these agonists significantly ($P>0.05$) affected thrombin-induced platelet accumulation in the head (Table 3). However, the D_1/D_2 agonists Z10997A and Z11410 produced a thrombocytopaenic effect (resulting in a non-significant reduction in platelet accumulation in the cerebral vasculature, Table 3 and Figure 2) by potentiating the initial accumulation of platelets in the lung following thrombin administration. These effects were only observed at doses higher than the intended therapeutic doses. Z11410 (0.1 μg kg^{-1} min^{-1}) significantly ($P<0.05$) increased platelet accumulation in the lung following thrombin (Figure 3). At lower doses of Z11410 (0.01 μg kg^{-1} min^{-1}) potentiation of platelet accumulation in the pulmonary vasculature did not occur and the effects in the cerebral circulation were reduced.

Table 1 Results of functional studies of D_1- and D_2-receptor, α_1- and α_2-adrenoceptor activity (pD_2) of dopamine agonists

	D_1	D_2	α_1	α_2
Dopamine	6.61	7.80	4.50	6.50
FDPM	7.83	NA	$pA_2=5.27$	Antag
DPDA	5.38	7.42	NA	<4
Z1046	6.85	9.84	$pA_2=6.5$	6.4
Z10997A	8.50	10.90	6.74	7.64
Z11410	8.50	10.52	5.63	6.30

The compounds tested were dopamine, fenoldopam (FDPM), dipropyldopamine (DPDA), Z1046, Z10997 and Z11410 (full chemical structure given in text). NA=no activity.

Table 2 Effect of intravenous (i.v.) infusion of dopamine and dopamine agonists upon ADP-induced platelet accumulation in the pulmonary vasculature of the rabbit

Treatment	*Before*	*During*	*Basal*
Saline	47±2	46±3	−3±0.3
Dopamine 30 μg kg^{-1} min^{-1}	57±1	55±1	−3±1
Dopamine 100 μg kg^{-1} min^{-1}	48±9	50±11	−2±1
Dopamine 2 mg kg^{-1} min^{-1}	44±4	34±5*	−1±1
FDPM 30 μg kg^{-1} min^{-1}	45±1	42±2	−4±1
Z1046 30 μg kg^{-1} min^{-1}	34±3	32±5	−5±2
DPDA 30 μg kg^{-1} min^{-1}	40±4	40±5	+1±0.4
Z10997A 10 μg kg^{-1} min^{-1}	48±5	41±8	−2±0.7

Results are expressed as maximum percentage increase in counts (mean±s.e.mean; $n=5$ except saline, where $n=7$) above baseline values before and after drug infusion, following administration of ADP (20 μg kg^{-1}, i.v.). *Significantly different from first response ($P<0.01$). Basal values refer to changes in pulmonary baseline values above (+) or below (−) initial count during drug infusion before the administration of ADP.

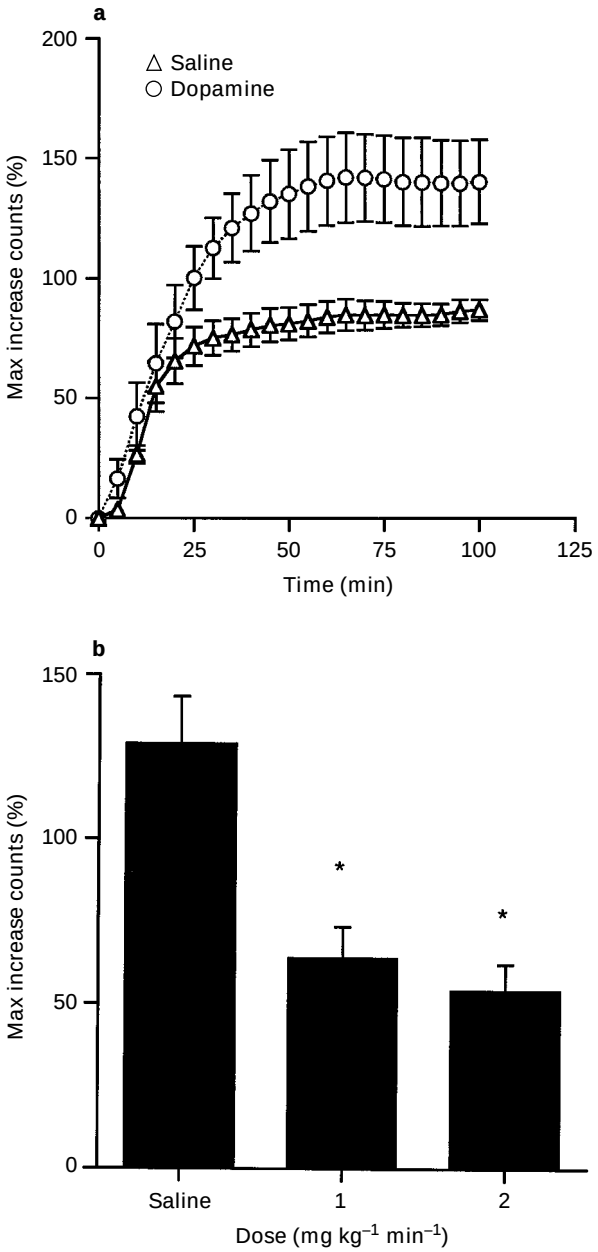

Figure 1 Effects of intracarotid (i.c.) dopamine infusions on thrombin-induced platelet accumulation in the cerebral vasculature of rabbits. Changes in ^{111}In-labelled platelet accumulation were recorded following i.c. injection of thrombin (90 u kg^{-1}) at time 0. Animals were infused with saline or dopamine commencing 40 min before the injection of thrombin and continuing throughout the recording period. Responses are expressed as % change in ^{111}In counts from stable baseline values and mean and s.e.mean (vertical lines) values are shown. In (a), % changes in ^{111}In levels have been plotted against time in animals infused with saline or dopamine 100 μg kg^{-1} min^{-1}. In (b), responses have been expressed as peak (maximum) % changes in counts in animals infused with saline or dopamine 1 or 2 mg kg^{-1} min^{-1}; $n=5$ animals/group throughout. *$P<0.05$ compared to animals infused with saline.

Table 3 Effect of intracarotid (i.c.) infusion of dopamine agonists upon thrombin-induced platelet accumulation in the cerebral vasculature of the rabbit

Drug	*Dose* ($\mu g\ kg^{-1}\ min^{-1}$)	*Max increase in head*	*Max increase in lung*	*Basal*
Saline		89±5	28±3	−2±1
Dopamine	30	93±10	45±9	0±1
Z1046	30	104±5	24±7	−1±3
FDPM	30	97±14	21±4	+1±1
DPDA	30	102±4	32±3	−2±2
Z10997A	1	108±9	45±3	0±2
Z10997A	10	93±9	38±14	−7±1
Z10997A	25	64±10	50±9	−8±2
Z11410	0.01	121±12	32±6	−1±1
Z11410	0.1	103±17	54±7*	−1±1

Results are expressed as maximum % increase in counts above baseline values following administration of thrombin (90 ug kg^{-1}, i.c.). *Significantly different from saline control values ($P<0.05$). Basal values refer to changes in cerebral baseline values above (+) or below (−) initial count during drug infusion before administration of thrombin.

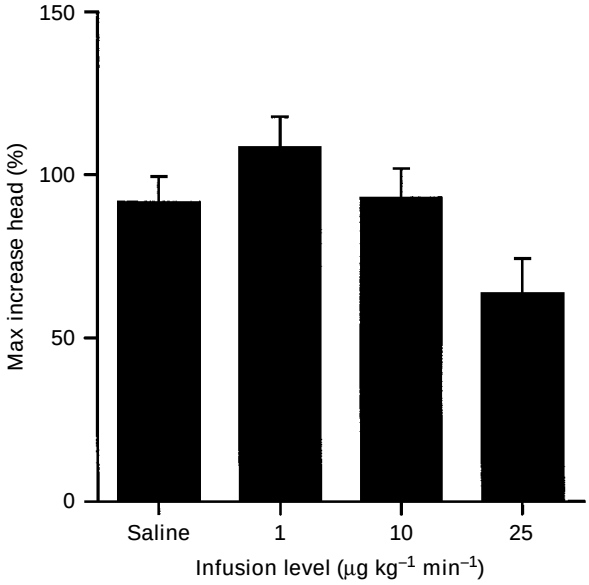

Figure 2 Effect of i.c. Z10997A infusion on thrombin-induced platelet accumulation in the cerebral vasculature of rabbits. Changes in ^{111}In-labelled platelet accumulation in the cerebral vasculature were recorded following i.c. injection of thrombin (90 u kg^{-1}). Animals were infused with saline or Z10997A (1, 10 or 25 $\mu g\ kg^{-1}\ min^{-1}$) commencing 40 min before the injection of thrombin and continuing for the duration of the recording period. Responses are expressed as maximum % change from stable baseline values and mean±s.e.mean values are shown; $n=5$ animals/group.

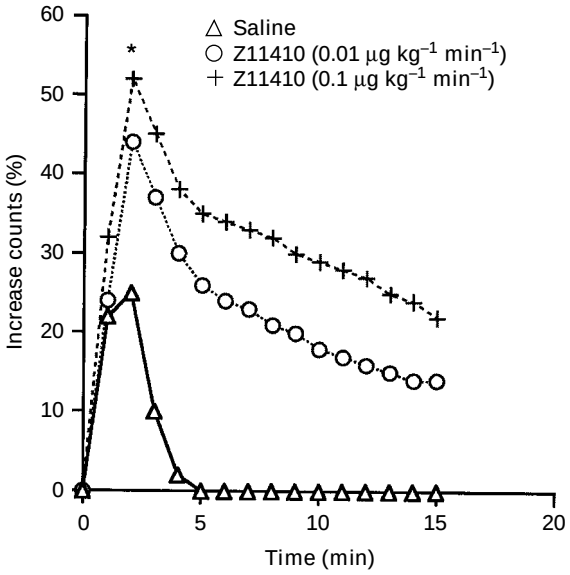

Figure 3 Effect of i.c. Z11410 upon thrombin-induced platelet accumulation in the pulmonary vasculature of rabbits. Changes in ^{111}In-labelled platelet accumulation in the pulmonary vasculature were recorded following i.c. injection of thrombin (90 u kg^{-1}) at time 0. Animals were infused with saline or Z11410 0.01 $\mu g\ kg^{-1}\ min^{-1}$ or 0.1 $\mu g\ kg^{-1}$ commencing 40 min before the injection of thrombin and continuing throughout. Results are expressed as % change in ^{111}In counts above stable baseline values; $n=5$ animals/group. *$P<0.05$ compared to saline controls.

Discussion

The results presented here extended previous *in vitro* findings (Braunstein *et al*., 1977) that low doses of dopamine can potentiate and higher doses inhibit thrombin induced platelet aggregation. It is not clear why dopamine (2 mg $kg^{-1}\ min^{-1}$) inhibited both ADP- and thrombin-induced platelet aggregation in the pulmonary and cerebral vasculature, respectively, whereas a lower dose of dopamine potentiated thrombin-induced platelet accumulation in the cerebral vasculature, but had no effect on ADP in the lung. However, thrombin induces a multitude of effects including fibrin deposition, vasoconstriction and tissue damage in addition to its effects on platelet aggregation. It is possible, therefore, that dopamine has an action on thrombin-induced aggregation that does not occur when a pure platelet agonist such as ADP is administered. In addition, pharmacological effects in the lung vasculature are known to differ from those in the cerebral vasculature (May *et al*., 1991).

The observation of two distinct effects (potentiation and inhibition of platelet aggregation) by a single agonist suggests the involvement of two or more signal transduction systems with different affinities for dopamine.

Dopamine acts at both α- and β-adrenoceptors in addition to dopamine receptors. Potentiation of platelet aggregation *in vitro* is thought to occur via α-adrenoceptors (Ahtee & Michal, 1972). Although inhibitory effects could be mediated via β-adrenoceptors, it has been shown that inhibition of agonist-induced aggregation by dopamine *in vitro* does not occur via β-adrenoceptors but is more likely mediated by D_1-like receptors (De Keyser *et al*., 1988).

The suggestion of the involvement of D_1-like receptors in the inhibition of platelet aggregation provided the rationale for the use of specific dopamine agonists in the experiments presented here. None of the dopamine agonists used were effective in inhibiting thrombin or ADP-induced aggregation at the doses tested, although the most potent D_1/D_2 agonist (Z11410) was shown to produce a significant potentiation of platelet accumulation in the lung at high doses. This potentiation means that platelet disaggregation is prolonged, leading to increased numbers of platelets in the lung 15 min or more after administration of thrombin (Figure 3) and hence fewer platelets in the cerebral vasculature. This effect demonstrates the importance of measuring platelet counts in more than one anatomical site, in this case the head and the lung, in order to distinguish between thrombocytopaenic effects of the drug and true inhibition of platelet aggregation.

We have not yet determined the mechanism by which enhancement of thrombin induced pulmonary platelet accumulation by compounds such as Z11410 occurs. If potentiation of pulmonary platelet accumulation is shown to occur via α-adrenoceptors, then future experiments could involve the use

of better, more specific D_1 and D_2 agonists, as those tested here have significant adrenoceptor activity (Table 1). On the other hand, if potentiation of platelet accumulation in the pulmonary vasculature occurs via a dopaminergic route then this has implications for the potential clinical uses of dopamine agonists.

It should be noted that dopamine agonists have cardiohaemodynamic effects which must be considered when determining the effects of these compounds on platelet accumulation *in vivo*. Dopamine agonists act as antihypertensive agents. In the anaesthetized dog, Z1046 decreases blood pressure and systemic vascular resistance and increases renal and femoral blood flow (Zambon Group, unpublished data). These haemodynamic effects may partly explain some of the observations presented here, for example the shift in baselines during drug infusion and thrombocytopaenic effects in the cerebral vasculature.

In conclusion, we have demonstrated that, depending upon the dose employed, dopamine can potentiate or inhibit thrombin-induced platelet accumulation *in vivo*. The selective dopamine agonists tested did not inhibit cerebrovascular platelet accumulation induced by thrombin, but at high doses potentiated platelet accumulation in the lung via an as yet unknown mechanism.

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(Received April 10, 1997
Revised June 23, 1997
Accepted July 10, 1997)