Biochemical Pharmacology, Vol. 32, No. 5, pp. 765–771, 1983.
Printed in Great Britain.

0006-2952/83/050765-07 $03.00/0

PLATELET-MEDIATED VASCULAR CONTRACTIONS

INHIBITION BY FLUNARIZINE, A CALCIUM-ENTRY BLOCKER

FRED DE CLERCK* and JAN M. VAN NUETEN

Laboratory of Haematology and Department of Pharmacology, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium

(*Received* 18 *May* 1982; *accepted* 23 *August* 1982)

Abstract—Flunarizine, a calcium (Ca^{2+})-entry blocker, selective for vascular tissues, inhibits in a concn-dependent way the contraction of isolated rat caudal artery preparations induced by mediators derived from thrombin-stimulated rat platelets. This inhibition is slow in onset and is of prolonged duration. Specific measurements and pharmacological analysis show 5-hydroxytryptamine (5HT) and thromboxane A_2 (TXA_2) to be the main mediators involved. Comparison with exogenously added agonists shows amplification between 5HT and TXA_2 at the level of the vascular smooth muscle cells. Combined treatment with ketanserin, a selective $5HT_2$ receptor antagonist, and suprofen, a fatty acid cyclo-oxygenase inhibitor, shows that flunarizine inhibits the 5HT-induced as well as the prostaglandin-induced components of the contraction. The compound does not affect the release of 5HT from the platelet and does not interfere with the biosynthesis of TXA_2 from endogenous platelet arachidonic acid; it reduces the amounts of TXB_2 and HHT and increases the production of HETE from exogenous [^{14}C]arachidonic acid by washed rat platelets.

Flunarizine {1-cinnamyl-4-[bis(*p*-fluorophenyl)-methyl]piperazine dihydrochloride} is an inhibitor of transmembrane Ca^{2+}-influx-induced vasoconstriction of peripheral and cerebral blood vessels, with little effect on myogenic activity and Ca^{2+}-dependent myocardial contractility [1–4]. In animal models the compound provides protection against ischemic cerebral cell damage [5], corrects cortical blood flow reduction and reduces the raised cerebral vascular resistance following transient cerebral ischemia induced by full circulatory arrest [6].

Accumulation of vasoconstrictor prostaglandins, e.g. thromboxane A_2 (TXA_2) and $PGF_{2\alpha}$, and 5-hydroxytryptamine (5HT) released from endogenous substrates or from platelets may contribute to the 'no-reflow phenomenon' subsequent to such a transient cerebral ischemia or to the vascular changes seen during a migraine attack [7–9].

Since blood platelets upon suitable stimulation release/synthesize such vasoactive mediators [10, 11], we studied the effect of flunarizine on platelet-mediated contractions of isolated rat caudal artery preparations.

MATERIALS AND METHODS

Isolated blood vessels

Spiral strips (4–5 cm length, 2 mm width) of the caudal artery of rats (Wistar, male, 200–250 g) were mounted vertically with a pre-load of 1.5 g in an organ chamber containing Krebs–Henseleit solution (20 ml) at 37° and continuously gassed with a mixture of 95% O_2 and 5% CO_2. Isometric contractions were continuously recorded by a Statham UC_2 force transducer [2, 12, 13]. The reactivity of the vascular preparations was assessed by measuring their contractile response to exogenously added 5HT (4.5×10^{-7}, 2.25×10^{-7} and 1.125×10^{-7} M), 1-norepinephrine hydrochloride (1-norepin) (4.7×10^{-7} M) and bovine thrombin (0.002 N.I.H. U/l).

Platelet suspensions

Rat (Wistar, male, 200–250 g) platelet suspensions ($1 \times 10^6/\mu l$) in Tyrode's solution without $CaCl_2$ or $MgCl_2$ containing 0.1% bovine serum albumin (Aldrich) and 1% glucose (pH 7.35), or in Krebs–Henseleit solution without $CaCl_2$ (pH 7.35), were prepared from acid–citrate–dextrose anticoagulated (2 vols/8 vols of blood) venous blood as previously described [14].

Platelet prostaglandin biosynthesis

Malondialdehyde (MDA). MDA production by washed rat platelets as an indicator for prostaglandin biosynthesis in the presence or absence of flunarizine and suprofen was evaluated spectrophotometrically essentially according to McMillan *et al.* [15]. Briefly, rat platelets (4×10^8/ml) suspended in Krebs–Henseleit solution, without $CaCl_2$, pre-incubated for 5 min at 37° with solvent or compound solution, were stimulated by the addition of $CaCl_2$ (1×10^{-3} M) and thrombin (1 or 20 N.I.H. U/ml). After 30 min at 37° the reaction was terminated by the addition of trichloroacetic acid (5% w/v); after extraction the thiobarbituric acid reaction for MDA was performed and the fluorescence at 553 nm after excitation at 510 nm was measured. Results are expressed as nM MDA/10^9 platelets/30 min.

Thromboxane synthesis. In a similar test system the production of TXA_2, estimated from the amounts

* To whom correspondence should be addressed at: Laboratory of Haematology, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium.

of thromboxane B_2 (TXB_2), by washed rat platelets was assayed. In these experiments the reaction was stopped by the addition of EDTA (0.14 mg%) and suprofen (1×10^{-5} M) and cooling of the sample. Thereafter platelet-free plasma was prepared by centrifugation of the reaction mixture (10,000 $g \times$ 5 min).

The amounts of TXB_2 generated were assayed by a radioimmunoassay (RIA) based on that proposed by Ferraris *et al.* [16] using a [^{3}H]TXB_2 RIA kit (New England Nuclear) as previously described [17]. Results are expressed as μg/l of supernatant.

Possible interference of flunarizine with the RIA of TXB_2 was tested by the addition of the compound to preformed TXB_2 before performance of the RIA.

Platelet [^{14}C]arachidonic acid metabolism. [^{14}C]Arachidonic acid (230 ng/ml, 100,000 cpm/ml, 0.1 ml) was added to the washed rat platelets (0.9 ml), suspended in Krebs–Henseleit solution without $CaCl_2$ ($5 \times 10^5/\mu$l), pre-incubated for 5 min at 37° with 1 μl of solvent or compound solution. After 30 min of reaction at 37°, the mixture was acidified to pH 3 with 2 N citric acid and extracted with ethyl acetate (2 × 2 vols). The organic layers were combined, dried under nitrogen, redissolved in chloroform : methanol (2 : 1) and applied to a TLC plate (Merck, Silica gel 60F25 4) together with authentic standards (arachidonic acid, $PGF_{2\alpha}$ and PGE_2; 10 μg each), The mixture was chromatographed in chloroform : methanol : acetic acid : water (90 : 6 : 1 : 0.6). After localization of the radioactive zones by radiochromatogram scanning (Berthold LB 2723 Thin-layer Chromatogram Scanner), the amount of radioactive metabolites was determined by conventional liquid scintillation counting and expressed as the percentage of the total amount of radioactivity recovered using previously described techniques [18, 19].

Release of platelet 5HT

Platelet-bound 5HT in the pool of washed platelet suspension was determined spectrofluorimetrically on $ZnSO_4$-deproteinized samples [20]. In some experiments the platelets were labelled with radioactive 5HT by the addition of [^{14}C]5HT (1.72×10^{-7} M); after an uptake period of at least 60 min at room temperature (20–22°), 75–85% of the added radioactivity was stored in the platelets [14].

After (2 min) thrombin stimulation of the radiolabelled platelets diluted in the organ chamber to 2.5×10^{10}/l, 1-ml samples were collected in 4 ml of ice-cold EDTA (0.5%) in NaCl (0.15 M) and centrifuged to a pellet (5 min × 1300 g). After washing in the same solution the pellet was solubilized in NaOH (0.5 N); radioactivity was then determined by conventional liquid scintillation counting [14, 17]. The percentage of released [^{14}C]5HT, in relation to the total 5HT content of the platelet suspension, was used to calculate the actual concentration of free 5HT released from the platelets into the bath fluid [10].

Platelet-mediated vascular contractions

The vascular strips were pre-incubated for 4 min with solvent or compound solution before the addition of 0.5 ml of the rat platelet suspension (2.5×10^{10}/l) in Tyrode's without $CaCl_2$ and $MgCl_2$ to the organ chamber containing 20 ml of Krebs–Henseleit solution. One minute thereafter 0.5 ml of thrombin (0.002 N.I.H. U/l) was added to produce stimulation of the platelets. The total contraction occurring after addition of the platelets and their subsequent challenge with thrombin, expressed in g, was used for data processing [10].

In a group of solvent experiments, 1-ml volumes of the organ chamber fluid were sampled 1 min after the addition of thrombin and were processed as previously described [14, 17, 21] for the determination of TXB_2 by RIA and of platelet-released [^{14}C]5HT.

In a first group of experiments a platelet-mediated vascular contraction in the presence of solvent was performed before the incubation of flunarizine or its solvent with the vascular strip. Five minutes after the introduction of the compound another reaction was induced. After 8 min of contact with the vascular strip, flunarizine was removed from the organ chamber by repeated wash-outs. Consecutive platelet-mediated vascular contractions, spaced by a wash-out, were then performed 25, 45, 65, 85 and 105 min after the addition of the compound to the organ chamber.

In a second group of experiments, the compound was introduced into the organ chamber in both reaction and washing fluids. Subsequent contractions in the continuous presence of the compound or its solvent then were induced 17, 37, 57, 77 and 97 min after the initiation of incubation of the compound with the vascular strip.

In a third group of experiments, platelet-mediated vascular contractions were induced after 5 min of incubation of the vascular strips with either ketanserin (1×10^{-7} M), suprofen (9.6×10^{-6} M), a combination of both compounds or their appropriate solvents.

In a last group of experiments, platelet-mediated vascular contractions were measured after incubation of the artery preparation with solvent, after 37 min of continuous contact with flunarizine (6.2×10^{-7} M) and after 57 min of continuous contact with flunarizine (6.2×10^{-7} M) in the presence of ketanserin (1×10^{-7} M, 5-min contact) or suprofen (9.6×10^{-6} M, 5-min contact).

Chemicals

5HT creatinine sulphate (Serva) and l-norepin (Aldrich) were dissolved in distilled water containing 0.026 mM Ca-EDTA. [Side-chain-2-^{14}C]5HT creatinine sulphate [sp. act. 60 mCi/mmole (147 μCi/mg) (the Radiochemical Centre, Amersham, U.K.)] was diluted in H_2O and stored at −20°. Bovine thrombin (Topostasine®, Roche) was dissolved in ice-cold 0.15 M NaCl. [^{14}C]Arachidonic acid [sp. act. 54.9 mCi/mmole (New England Nuclear)] was dissolved in ethanol and stored at −25°. Authentic arachidonic acid, $PGF_{2\alpha}$ and PGE_2 (Sigma) were all dissolved together in ethanol to a concn of 1 mg/ml. Suprofen (Janssen Pharmaceutica), EDTA (Merck) and $CaCl_2 \cdot 2H_2O$ (Merck) were dissolved in 0.15 M NaCl. For experiments on vascular contractions, flunarizine (Janssen Pharmaceutica) and ketanserin

(Janssen Pharmaceutica) were prepared as an aqueous solution containing 0.1 M tartaric acid (pH 3.1). Suprofen was dissolved in 2% sodium carbonate (pH 9.4). Solvent or compound solutions were added in 0.2-ml vols to the organ chamber fluid (20 ml). For experiments on isolated platelets both flunarizine and suprofen were dissolved in dimethylsulphoxide (DMSO). All concentrations are expressed as final ones in the reaction mixtures.

The millimolar composition of the Krebs–Henseleit solution was NaCl 118.1, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.1, and of the Tyrode's solution was NaCl 130, KCl 2.6, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 7.2, NaH_2PO_4 4, glucose 11.1.

Statistical evaluation

Results are expressed as means ± S.E.M. of the stated number of experiments performed on different preparations. Statistical evaluation was performed using the Student's *t*-test, P values ≤ 0.05 being considered as statistically significant.

RESULTS

Platelet prostaglandin production

MDA. The incubation of flunarizine (5×10^{-6} M) with washed rat platelets (4×10^8/ml) did not affect the amount of MDA produced during a 30-min period at 37° after stimulation with thrombin at 1 or 20 U/ml (1 U/ml thrombin: DMSO = 1788.3 ± 42.2; flunarizine = 1605.8 ± 50.2; 20 U/ml thrombin: DMSO = 1536.5 ± 38.1; flunarizine = 1519 ± 32.8 nM MDA/10^9 platelets/30 min, N = 6). On the contrary, the cyclo-oxygenase inhibitor suprofen (1×10^{-5} M) completely inhibited the formation of MDA at both thrombin concentrations (1 U/ml thrombin: suprofen = 0 ± 0; 20 U/ml thrombin: suprofen = 0 ± 0 nM MDA/10^9 platelets/30 min, N = 6).

Thromboxane synthesis

At concns up to 5×10^{-6} M flunarizine did not significantly modify the amount of TXB_2 produced by washed rat platelets during 30 min at 37° after stimulation with thrombin at 1 or 20 U/ml (1 U/ml thrombin: DMSO = 5.68 ± 0.88; flunarizine = 4.37 ± 0.21; 20 U/ml thrombin; DMSO = 5.06 ± 0.11; flunarizine = 5.18 ± 0.11 TXB_2 μg/l, N = 4). In this concn range flunarizine did not interfere with the RIA of preformed TXB_2.

Platelet [^{14}C]arachidonic acid metabolism

Pre-exposure to flunarizine (5×10^{-6} M) for 5 min at 37° did not significantly affect the relative amounts of the cyclo-oxygenase products $PGF_{2\alpha}$, PGE_2 and PGD_2 formed from [^{14}C]arachidonic acid by washed rat platelets during a 30-min incubation at 37°; however, the compound induced a slight but significant reduction of TXB_2 (−30.6%) and of 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (−26.3%) production and an increase (+113.3%) of the lipoxygenase product 12-L-hydroxy-5,8,10,14-eicosatetranoic acid (HETE) (Table 1).

Release of platelet 5HT

Measurements of radioactivity in the cell-free supernatant showed that [^{14}C]5HT labelled rat platelets, diluted to 2.5×10^{10}/l in the organ chamber containing Krebs–Henseleit solution, released 71.92 ± 0.58% (N = 8) of their bound radioactivity upon stimulation with thrombin (0.002 N.I.H. U/ml). In these experimental conditions flunarizine (5×10^{-6} M) did not affect the percentage of release induced by such thrombin stimulation (76.08 ± 1.04%, N = 8).

Table 1. Effect of flunarizine on rat platelet [^{14}C]arachidonic acid metabolism

Products*	Treatment†		
	Solvent	Flunarizine	Boiling
Phospholipids	22.78 ± 1.77	19.35 ± 3.08	0.65 ± 0.11‡
$PGF_{2\alpha}$	3.45 ± 0.39	2.99 ± 0.23	0.09 ± 0.01‡
TXB_2	22.85 ± 1.67	15.84 ± 1.42‡	0.12 ± 0.01‡
PGE_2	2.84 ± 0.15	2.51 ± 0.30	0.08 ± 0.01‡
PGD_2	2.43 ± 0.18	1.80 ± 0.16	0.12 ± 0.02‡
HHT§	11.75 ± 0.45	8.66 ± 0.82‡	0.22 ± 0.04‡
HETE‖	14.82 ± 2.01	31.62 ± 5.20‡	0.32 ± 0.06‡
Non-metabolized arachidonic acid	12.98 ± 0.96	11.98 ± 2.90	96.91 ± 0.17‡
Rest	6.08 ± 0.57	5.24 ± 1.03	1.45 ± 0.10‡
Total radioactivity (cpm)	62,708 ± 771.17	65,553 ± 1733.68	72,143.5 ± 1652.1

* Fractions, recovered after TLC separation, expressed as percentages of total radioactivity. Mean ± S.E.M.

† Washed rat platelets (4.5×10^8/ml) in contact for 5 min at 37° with solvent (0.1% DMSO, N = 8) or flunarizine (5×10^{-6} M) (N = 4) and incubated for 30 min at 37° with [^{14}C]arachidonic acid (230 ng/ml, 100,000 cpm/ml). Boiled platelet samples (N = 4) spiked with [^{14}C]arachidonic acid to quantify tailing on TLC plate.

‡ P ≤ 0.05 vs solvent; Student's *t*-test (two-tailed probability).

§ 12-L-Hydroxy-5,8,10-hepatadecatrienoic acid.

‖ 12-L-Hydroxy-5,8,10,14-eicosatetranoic acid.

Platelet-mediated vascular contractions

Comparison with exogenous agonists. The addition of rat platelets (2.5×10^{10}/l) to the organ chamber produced a slight contraction of the rat caudal artery preparations (Table 2). Subsequent stimulation of the platelets with thrombin (0.002 N.I.H. U/l), 1 min after their addition to the organ chamber produced a fast and reproducible further contraction of the vascular preparations (Table 2). Such thrombin stimulation produced 71.92 ± 0.58% (N = 8) release of platelet-bound 5HT resulting in a concn of $1.48 \pm 0.05 \times 10^{-7}$ M of free 5HT in the organ chamber. This activation produced TXB_2 levels of 4.34 ± 0.23 μg/l.

Despite the comparatively low levels of free 5HT derived from the platelets the extent of platelet-mediated contraction was superior to that produced by a three-fold higher concn of exogenously added 5HT. Thrombin alone produced a slight contraction of the artery preparation on its first addition but was without effect on the vascular tone on subsequent additions.

Inhibition by flunarizine

The incubation of the rat caudal artery preparation with flunarizine for 8 min, followed by repeated wash-outs resulted in a concn-dependent inhibition of the platelet-mediated vascular contractions (Fig. 1). The inhibitory effect of the compound after such a brief incubation progressively increased to reach a maximum at 25 min after the start of the initial contact. For the lower concns tested (6.2×10^{-7} and 3.1×10^{-7} M) the inhibition tended to level off slightly after the maximal effect as the number of consecutive wash-outs increased. The highest concentration (5×10^{-6} M) produced a maximal inhibition of 70%, maintained up to 105 min, even after repeated wash-outs.

The continuous presence of flunarizine (6.2×10^{-7} and 5×10^{-6} M) in both the reaction and wash fluids resulted in a progressively increasing inhibition of the platelet-mediated vascular contractions, maximal inhibition of 72–79% being achieved with 5×10^{-6} M incubated for 37–77 min with the vascular preparations. Platelet-mediated vascular contractions produced in the presence of solvent remained within the same range for the duration of the experiment (Table 3).

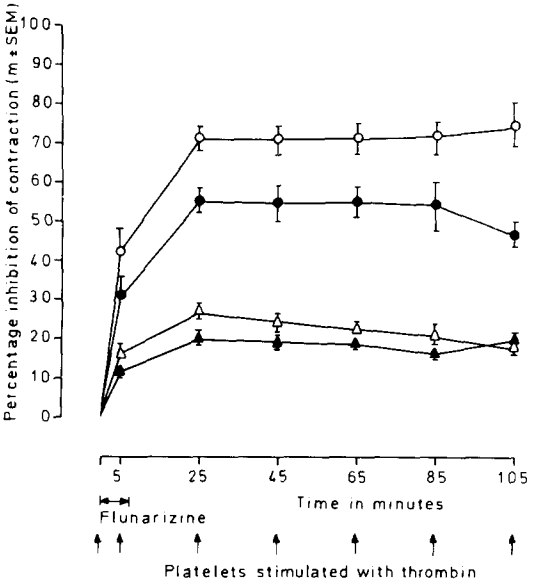

Fig. 1. Concn-dependent inhibition by flunarizine (▲ = 3.1×10^{-7} M, △ = 6.2×10^{-7} M, ● = 1.25×10^{-6} M, ○ = 5×10^{-6} M; contact 8 min) of the rat caudal artery contractions induced by washed rat platelets (2.5×10^{10}/l) stimulated with thrombin (0.002 N.I.H. U/l). Means ± S.E.M. of 6–11 experiments on different artery preparations.

Combination of flunarizine with other antagonists

(a) The incubation of the rat caudal artery preparation with ketanserin (1×10^{-7} M) for 5 min resulted in a partial inhibition (−51.9%) of the vascular contraction produced by thrombin-stimulated platelets (solvent = 0.856 ± 0.0592 g;

Table 2. Comparison of rat caudal artery contractions induced by exogenous agonists and by platelet-derived mediators

Treatment		Parameter‡		
		Contraction (g)	Platelet-released 5HT ($\times 10^{-7}$ M)	TXB_2 (μg/l)
5HT	1.12×10^{-7} M	0.341 ± 0.0374	—	—
	2.25×10^{-7} M	0.594 ± 0.0538§	—	—
	4.5×10^{-7} M	0.816 ± 0.0468§	—	—
1-Norepin	4.7×10^{-7} M	0.904 ± 0.0484	—	—
Thrombin (1 st)	0.002 U/l	0.200 ± 0.0204§	—	—
Platelets*		0.231 ± 0.0472§	—	—
Platelets + thrombin†		0.918 ± 0.0494	1.48 ± 0.05	4.3 ± 0.23

* Rat platelets (2.5×10^{10}/l) added to the organ chamber 1 min before thrombin. Contraction at 1 min after their addition.

† Rat platelets stimulated with thrombin (0.002 N.I.H. U/l) 1 min after their addition to the organ chamber. Total contraction 3 min after thrombin addition.

‡ Means ± S.E.M. of N = 22.

§ $P \leq 0.05$ vs platelets + thrombin; Student's *t*-test (two-tailed probability).

Table 3. Effect of continuous incubation of rat caudal arteries with flunarizine on the platelet-mediated vascular contractions

Contact time* (min)	Platelet-mediated vascular contraction†		
		Flunarizine	
	Solvent	6.2×10^{-7} M	5×10^{-6} M
0	0.927 ± 0.0798	0.872 ± 0.0808	0.800 ± 0.0982
5	0.922 ± 0.0742		—
17	—	0.635 ± 0.0494 (26.1 ± 2.8)	0.278 ± 0.0320 (−63.5 ± 4.55)
25	0.910 ± 0.0722	—	—
37	—	0.439 ± 0.0392 (48.84 ± 3.0)	0.211 ± 0.0316 (−72.50 ± 3.84)
45	0.882 ± 0.0714	—	—
57	—	0.377 ± 0.0358 (56.97 ± 2.37)	0.186 ± 0.0294 (−75.52 ± 3.60)
65	0.862 ± 0.061	—	—
77	—	0.352 ± 0.0344 (58.98 ± 3.05)	0.155 ± 0.0198 (−79.51 ± 2.70)
85	0.836 ± 0.0708	—	—
97	—	0.326 ± 0.033 (61.5 ± 3.14)	0.16 ± 0.0238 (−78.95 ± 2.77)
105	0.842 ± 0.0692	—	—

* Time (min) of continuous contact of flunarizine with the vascular preparation in reaction and wash period.

† Washed rat platelet (2.5×10^{10}/l) stimulated with thrombin (0.002 N.I.H. U/l).

Total contraction in g and percentage inhibition (between parentheses).

Means ± S.E.M. of 4 (solvent), 10 (6.2×10^{-7} M) and 6 (5×10^{-6} M) experiments on different preparations.

ketanserin = 0.412 ± 0.0954 g; N = 3). Similarly suprofen (9.6×10^{-6} M) produced partial inhibition (−22.5%) of the platelet-mediated vascular contraction (solvent = 0.942 ± 0.039 g; suprofen = 0.73 ± 0.0248 g; N = 3). Combined treatment of the arterial strips with ketanserin and suprofen produced substantially higher inhibition (−82.6%) than single-drug treatment (solvents = 0.936 ± 0.0632 g; ketanserin + suprofen = 0.163 ± 0.272 g; N = 3).

(b) The partial inhibition obtained with either ketanserin (1×10^{-7} M) or suprofen (9.6×10^{-6} M) or with combined treatment was further increased in the presence of flunarizine (6.2×10^{-7} M, 37 min of contact) (Table 4).

DISCUSSION

In agreement with earlier observations [10], the present study confirms that thrombin-stimulated rat platelets, as well as platelets activated on the de-endothelialized blood vessel, induce a substantial contraction of a peripheral artery such as the rat caudal artery preparation *in vitro*. Biochemical measurements of the various mediators involved, as well as pharmacological analysis showed 5HT and prostaglandins—in particular TXA_2—to be the principal mediators responsible for the platelet-mediated vasoconstriction, with a predominance for 5HT in this particular vascular preparation [10]. Indeed, in agreement with an earlier study [10], treatment of the vessel strips with ketanserin, a selective $5HT_2$ receptor antagonist without agonistic effect on isolated blood vessels [12, 22], reduces the platelet-mediated response [10] to a larger extent than the inhibition of platelet TXA_2 synthesis by suprofen, a fatty acid cyclo-oxygenase inhibitor [10, 23]. The

Table 4. Effect of flunarizine on the platelet-mediated rat caudal artery contraction in the presence of $5HT_2$ receptor blockade (ketanserin) and cyclo-oxygenase inhibition (suprofen)

Treatment	Platelet-mediated vascular reaction†	
	Contraction‡	Inhibition (%)
Solvent	0.795 ± 0.067§	—
Flunarizine (6.2×10^{-7} M)	0.327 ± 0.0418	59.6 ± 1.98
Flunarizine + ketanserin (1×10^{-7} M)	0.172 ± 0.0414§	79.4 ± 3.22
Flunarizine + suprofen (9.6×10^{-6} M)	0.133 ± 0.0316§	84.3 ± 2.4
Flunarizine + ketanserin + suprofen	0.046 ± 0.0058§	94.21 ± 0.59

* Rat caudal artery exposed to solvent and subsequently continuously exposed to flunarizine. Contractions recorded after 37 min of exposure and later. Ketanserin and suprofen added during 5 min in the presence of flunarizine.

† Rat platelets (2.5×10^{10}/l) stimulated with thrombin (0.002 N.I.H. U/l) in the organ chamber.

‡ Total contraction in g. Means ± S.E.M. (N = 8).

§ $P \leq 0.05$ vs flunarizine; Student's *t*-test (two-tailed probability).

non-serotonergic/non-prostaglandin part of the contraction may be mediated by adenine nucleotides rapidly released from aggregating platelets, by lipoxygenase products, PAF or other phospholipids [10]. The extent of the platelet-mediated vascular contraction cannot be explained by the single or additive effects of either 5HT or TXA_2 alone; measurements involving release of [^{14}C]5HT from labelled platelets and fluorimetric determination of the platelet-bound 5HT concns showed low levels (1.48×10^{-7} M) of platelet-derived, free 5HT in the organ chamber fluid in comparison with the concn (4.5×10^{-7} M) of exogenous 5HT required to produce a vascular contraction of comparable extent to the one induced by the activated platelets. The higher contractile response to low platelet-derived 5HT levels is not due to an additive effect of TXA_2 since inhibition of the prostaglandin biosynthesis by suprofen does not reduce the extent of the platelet-mediated contraction down to the level of the appropriate concn of exogenous 5HT alone. Similar to the amplifying effect of 5HT observed on other mediators such as norepinephrine, histamine, angiotensin II and $PGF_{2\alpha}$ [22, 24] the present study strongly suggests an amplification reaction between 5HT and TXA_2 as vasospastic agents.

At the thrombin concn and time period after its addition used in the present experiments the release of endogenous 5HT and the production of TXA_2 from/by the platelets are maximal (see results for platelet prostaglandin production and Refs. 25–27); in these conditions $5HT_2$ receptor inhibition with ketanserin did not reduce the platelet release of 5HT nor the formation of TXA_2; inhibition of TXA_2 production with suprofen, in the presence or absence of ketanserin, did not modify the release of 5HT from thrombin-stimulated platelets [10]. Therefore an amplification between 5HT and TXA_2 on the platelets rather than on the vascular smooth muscle cells seems unlikely in the present experimental conditions.

Thrombin has been demonstrated to relax several blood vessels *in vitro* [28–30]. On the contrary, some isolated blood vessels (e.g. dog pulmonary and mesenteric veins and basilar artery, rabbit vena cava and aorta) are constricted by the enzyme [31, 32]. Relaxation by thrombin of constricted canine femoral arteries was shown to be modulated by the vascular endothelium, independently from the formation of prostacyclin or lipoxygenase products [30]. Our present data demonstrate that thrombin also contracts the de-endothelialized rat caudal artery preparation on its first contact only; its mechanism of action is not clear but is not dependent upon the formation of TXA_2 or prostacyclin [10].

The platelet-mediated vascular contraction is inhibited in a concn-dependent way by flunarizine, a selective Ca^{2+}-entry blocker at the level of the vascular smooth muscle cell [1–3, 33]. As observed in previous studies on isolated peripheral arteries contracted by Ca^{2+}-challenge after K-depolarization [1–3] the inhibition by flunarizine has a gradual onset and a prolonged duration of action after repeated wash-outs. Specific measurements involving [^{14}C]5HT labelled platelets, MDA and TXB_2 production from endogenous, thrombin-liberated arachidonic acid show that flunarizine, in concns effective against vascular contractions, does not significantly affect the thrombin-induced release of 5HT from the platelets nor the production of the vasoconstrictive TXA_2 by these blood cells; therefore its primary site of action is on the activation of the vascular smooth muscle cell rather than on the production/release of mediators by the platelets. Only at the highest concn tested (5×10^{-6} M) did the compound slightly reduce the production of TXB_2 and HHT and increase that of HETE from exogenous [^{14}C]arachidonic acid by rat platelets. Lipoxygenase products have been suggested to be involved in the endothelium-dependent relaxation of vascular tissue induced by acetylcholine [30, 34], but not by thrombin [30]. Whether the increase of platelet lipoxygenase activity has some bearing on the anti-vasoconstrictor effect of flunarizine remains to be elucidated. Flunarizine reduces those parts of the platelet-mediated vascular contraction not affected by either the $5HT_2$ receptor antagonist ketanserin [10, 22] or the fatty acid cyclo-oxygenase inhibitor suprofen [10, 23]; therefore the compound is effective both against 5HT- and TXA_2-induced vasoconstriction. Such a finding is in agreement with the previously observed inhibition by flunarizine of vascular contractions induced by exogenous 5HT [13] and by exogenous $PGF_{2\alpha}$ [33]. Moreover it supports the involvement of a transmembrane influx of Ca^{2+} in the activation of vascular smooth muscle contraction by the monoamine and by some prostaglandins [13, 33, 35].

5HT and TXA_2 as potent contractile agents of smooth muscle of vascular and respiratory origin [12, 22, 36–41] may be implicated in pulmonary and cerebral disorders including pulmonary embolism and transient cerebral ischemia [7, 9, 39, 40]. The antagonism of flunarizine as a Ca^{2+}-entry blocker against these vasoactive mediators therefore may contribute to its beneficial effect [5, 6] on tissue perfusion in particular hypoxic reactions [6, 41].

Acknowledgements—We would like to thank Dr P. A. J. Janssen for his interest and stimulating criticism. The valuable technical assistance of E. Ghoos, Y. Somers and L. Van Gorp and the help of L. Geentjens and L. Leijssen in the preparation of the manuscript are greatly acknowledged. This study was partly supported by a grant from the Instituut tot Aanmoediging van Wetenschappelijk Onderzoek in Landbouw en Nijverheid (IWONL).

REFERENCES

1. J. M. Van Nueten and P. A. J. Janssen, *Archs int. Pharmacodyn. Thér.* **204**, 37 (1973).
2. J. M. Van Nueten, J. Van Beek and P. A. J. Janssen, *Archs int. Pharmacodyn. Thér.* **232**, 42 (1978).
3. J. M. Van Nueten and P. M. Vanhoutte, *Biochem. Pharmac.* **29**, 479 (1980).
4. M. Toyoda, S. Takagi, T. Seki, T. Takeoka and F. Gotoh, *J. Neurol. Sci.* **25**, 371 (1975).
5. A. Wauquier, D. Ashton, G. Clincke and J. Van Reempts, in *Cerebral Hypoxia in the Pathogenesis of Migraine* (Eds. F. Clifford-Rose and W. Amery), p. 139. Pitman Books, London (1982).
6. B. C. White, D. Gadzinski, P. J. Hockner, C. Krome and J. D. White, *Ann. Emerg. Med.* **22**, 118 (1982).
7. R. J. Gaudet and L. Levine, *Biochem. biophys. Res. Commun.* **86**, 893 (1979).

8. J. H. Dougherty, D. E. Levy and B. B. Weksler, *Neurology* **29**, 1460 (1979).
9. E. Hanington, *Lancet* **II**, 501 (1978).
10. F. De Clerck and J. M. Van Nueten, *Thromb. Res.* **27**, 713 (1982).
11. E. F. Ellis, O. Oelz, L. Jacson Roberts, II, N. A. Payne, B. J. Sweetman, A. S. Nies and J. A. Oates, *Science* **193**, 1135 (1976).
12. J. M. Van Nueten, in *5-Hydroxytryptamine in Peripheral Reactions* (Eds. F. De Clerck and P. M. Vanhoutte), p. 77. Raven Press, New York (1982).
13. J. M. Van Nueten and P. M. Vanhoutte, *Angiology* **32**, 476 (1981).
14. F. De Clerck and R. S. Reneman, *Naunyn-Schmiedeberg's Archs Pharmac.* **278**, 261 (1973).
15. R. M. McMillan, D. E. MacIntyre and J. L. Gordon, *Thromb. Res.* **11**, 425 (1977).
16. V. Ferraris, J. B. Smith and M. J. Silver, *Thromb. Homeost.* **38**, 20 (1977).
17. F. De Clerck, J. L. David and P. A. J. Janssen, *Agents and Actions* **12**, 388 (1982).
18. P. Needleman, A. Raz, J. A. Ferrendelli and M. Minkes, *Proc. natn. Acad. Sci. U.S.A.* **74**, 1716 (1977).
19. J. R. Beetens, M. Claeys and A. G. Herman, *Biochem. Pharmac.* **30**, 2811 (1981).
20. F. Geeraerts, L. Schimpfessel and R. Crokaert, *Experientia* **30**, 837 (1974).
21. F. De Clerck, J. L. David and P. A. J. Janssen, in *5-Hydroxytryptamine in Peripheral Reactions* (Eds. F. De Clerck and P. M. Vanhoutte), p. 83. Raven Press, New York (1982).
22. J. M. Van Nueten, J. Van Beek, R. Xhonneux, T. Verbeuren and P. M. Vanhoutte, *J. Pharmac. exp. Ther.* **218**, 217 (1981).
23. J. M. Van Nueten, J. Hoebeke, F. De Clerck, F. Awouters and P. A. J. Janssen, *Archs int. Pharmacodyn. Thér.* **220**, 297 (1978).
24. J. M. Van Nueten, P. A. J. Janssen, W. De Ridder and P. M. Vanhoutte, *Eur. J. Pharmac.* **77**, 281 (1982).
25. G. Ragtar, M. Livio, J. Merino and G. De Gaetano, *Thromb. Haemostas.* **44**, 49 (1980).
26. M. A. Packham, M. A. Gullione, P. L. Chang and J. F. Mustard, *Am. J. Physiol.* **225**, 98 (1973).
27. M. B. Zucker, *Thromb. Diath. haemorrh.* **28**, 393 (1972).
28. R. Verhaeghe and W. Janssens, in *Vasodilatation* (Eds. P. M. Vanhoutte and I. Leusen), p. 141. Raven Press, New York (1981).
29. W. J. Janssens and R. H. Verhaeghe, *Blood Vessels* **19**, 126 (1982).
30. J. G. De Mey and P. M. Vanhoutte, *Archs int. Pharmacodyn. Ther.* **250**, 314 (1981).
31. K. O. Haustein and F. Markwardt, *Archs int. Pharmacodyn. Thér.* **163**, 393 (1966).
32. R. P. White, C. E. Chapbrau, M. Dugdale and J. T. Robertson, *Stroke* **11**, 363 (1980).
33. T. Godfraind and R. C. Miller, *Br. J. Pharmac.* **73**, 252 (1981).
34. R. F. Furchgott and J. V. Zadwadzki, *Nature, Lond.* **288**, 373 (1980).
35. D. F. Bohr, S. Greenberg and A. Bonaccorsi, in *Microcirculation* (Eds. G. Kaley and B. M. Altura), Vol. 2, p. 311. University Park Press, Baltimore (1978).
36. B. Samuelsson, G. C. Folco, E. Grandström, H. Kindahl and C. Malmsten, in *Advances in Prostaglandin and Thromboxane Research* (Eds. F. Coceani and P. M. Olley), Vol. 4, p. 1. Raven Press, New York (1978).
37. G. J. Dusting, S. Moncada and J. R. Vane, *Eur. J. Pharmac.* **49**, 65 (1978).
38. P. M. Vanhoutte, in *Microcirculation* (Eds. G. Kaley and B. M. Altura), Vol. 2, p. 181. University Park Press, Baltimore (1978).
39. H. Steinberg and A. Fisher, in *Serotonin in Health and Disease* (Ed. W. B. Essman), Vol. 5, p. 69. S. P. Medical & Scientific Books, New York (1979).
40. T. Utsunomiya, M. M. Krausz, D. Shepro and H. Hechtman, *Am. J. Physiol.* **241**, H766 (1981).
41. J. M. Van Nueten and F. De Clerck, in *Cerebral Hypoxia in the Pathogenesis of Migraine* (Eds. F. Clifford-Rose and W. Amery), p. 176. Pitman Books, London (1982).