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Heterogeneity of thromboxane A_2 (TP-) receptors: evidence from antagonist but not agonist potency measurements

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1 Thromboxane A_2 (TP-) receptors in human, rat and rabbit platelets and in smooth muscle of guinea-pig trachea, rat aorta and rabbit aorta have been characterized by measurement of the potencies of agonists and antagonists having considerable variations in chemical structure.

2 On each washed platelet system, eight prostanoids induced maximal irreversible aggregation (full agonists) and the potency ranking was EP 171 > STA_2 > 9,11-azo PGH_2 > 9,11-endoxy-10a-homo PGH_2 > U-46619 (standard) > PGH_2 = 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ > 16,16-dimethyl $PGF_{2\alpha}$. Correlations between the three platelet preparations for both absolute and relative potencies were good. On human platelets, STA_2, at concentrations above that required for maximum aggregation, exerted an inhibitory effect which was independent of its interaction with the TP-receptor.

3 Five prostanoids, EP 109, EP 167, EP 204, PTA_2 and 16,20-methano PTA_2, exhibited partial agonist activity on the platelet and smooth muscle preparations. There was good agreement between absolute potencies on the six preparations; on platelets potency was assessed from shape change measurements, since aggregation, when present, always showed a very shallow concentration-response relationship. The magnitude of the maximum response induced by each compound decreased in the order listed above, to the extent that 16,20-methano PTA_2 could be treated as a pure antagonist.

4 With U-46619 as agonist, the pA_2 values of seven antagonists were found to be very similar on human and rat platelets. The potency ranking was EP 169 > AH 23848 > EP 092 > ONO 11120 > EP 115 = 16,20-methano PTA_2 > BM 13177. There was a similar trend on rabbit platelets but pA_2 values were 1.0–1.5 log units smaller; the exception was BM 13177 which had similar affinities. The antagonism produced by EP 169 and AH 23848 was surmountable on rabbit platelets but not on human and rat platelets.

5 None of the antagonists was highly potent on the rabbit aorta (pA_2 values < 7.5 by Schild analysis). Affinities on the guinea-pig trachea and the rat aorta were higher and in the same range as those obtained for human and rat platelets. However the correlations of pA_2 values between any pair of smooth muscle preparations and between any pair of platelet/smooth muscle preparations were either weak or not significant ($P > 0.05$).

6 The excellent agreement for both full and partial agonist potencies between the six preparations provides no evidence for TP-receptor subtypes and further suggests that the agonist recognition sites of the TP-receptors could be very similar, if not identical, in nature. In contrast, the different antagonist affinities found in this and other published studies indicate heterogeneity of TP-receptors. However, classification into TP_1-, TP_2-receptors, etc. on the basis of the limited antagonist data available does not appear appropriate at this time.

Introduction

This paper addresses the possible existence of subtypes of the thromboxane A_2 (TP-) receptor. Our early *in vitro* studies with the TP-receptor antagonist EP 045 revealed a less effective block of the contractile actions of TXA_2 and U-46619 (a stable PGH_2 analogue, Figure 1) on rabbit aorta compared with guinea-pig trachea and dog saphenous vein (Jones *et al.*, 1982). In a preliminary report Anderson & MacIntyre (1982) also showed that EP 045 and several other TP-receptor antagonists were less potent as inhibitors of U-46619-induced aggregation in rabbit platelet-rich plasma (PRP) compared with human PRP. More extensive studies (Narumiya *et al.*, 1986) have shown that the TP antagonist, ONO 11120 (Katsura *et al.*, 1983) (Figure 1), is a less effective inhibitor of aggregation induced by STA_2 (a stable TXA_2 analogue, Figure 1) in rabbit PRP than in human PRP. In addition, radiolabelled I-PTA-OH, a close relative of ONO 11120, was found to bind with high affinity to TP-receptors on human and dog washed platelets but not rabbit washed platelets. In order to throw light on these potential differences between TP-receptors, we decided to measure pA_2 values for a number of TP antagonists on rabbit aorta and rabbit washed platelets and to compare these with corresponding values for guinea-pig trachea, rat aorta, and human and rat washed platelets.

STA_2 is a highly potent agonist at TP-receptors in smooth muscle with EC_{50} values in the 1–5 nM range (Mais *et al.*, 1985; Toda *et al.*, 1986). Its aggregatory potency appears to be lower: EC_{50} = 0.4 μM (Katsura *et al.*, 1983) and 1.1 μM (Mais *et al.*, 1985) for human PRP, 0.68 μM (Mais *et al.*, 1985) for dog PRP and 8 μM for rabbit PRP (Narumiya *et al.*, 1986). Although plasma protein binding in PRP could dramatically reduce the potency of this highly lipophilic molecule, we felt that true potency differences might be involved, particularly with respect to the rabbit platelet. We have therefore compared the potencies of STA_2 and seven other full agonists on washed platelets from man, rat and rabbit.

Finally we have compared the activities of several partial agonists, including the novel prostanoids EP 167 and EP 204 (Figure 1), on the platelet and smooth muscle preparations.

Methods

Washed platelet preparations

Blood was collected into acid-citrate-dextrose (ACD) anticoagulant (1 vol : 5 vol blood) from a forearm vein of human

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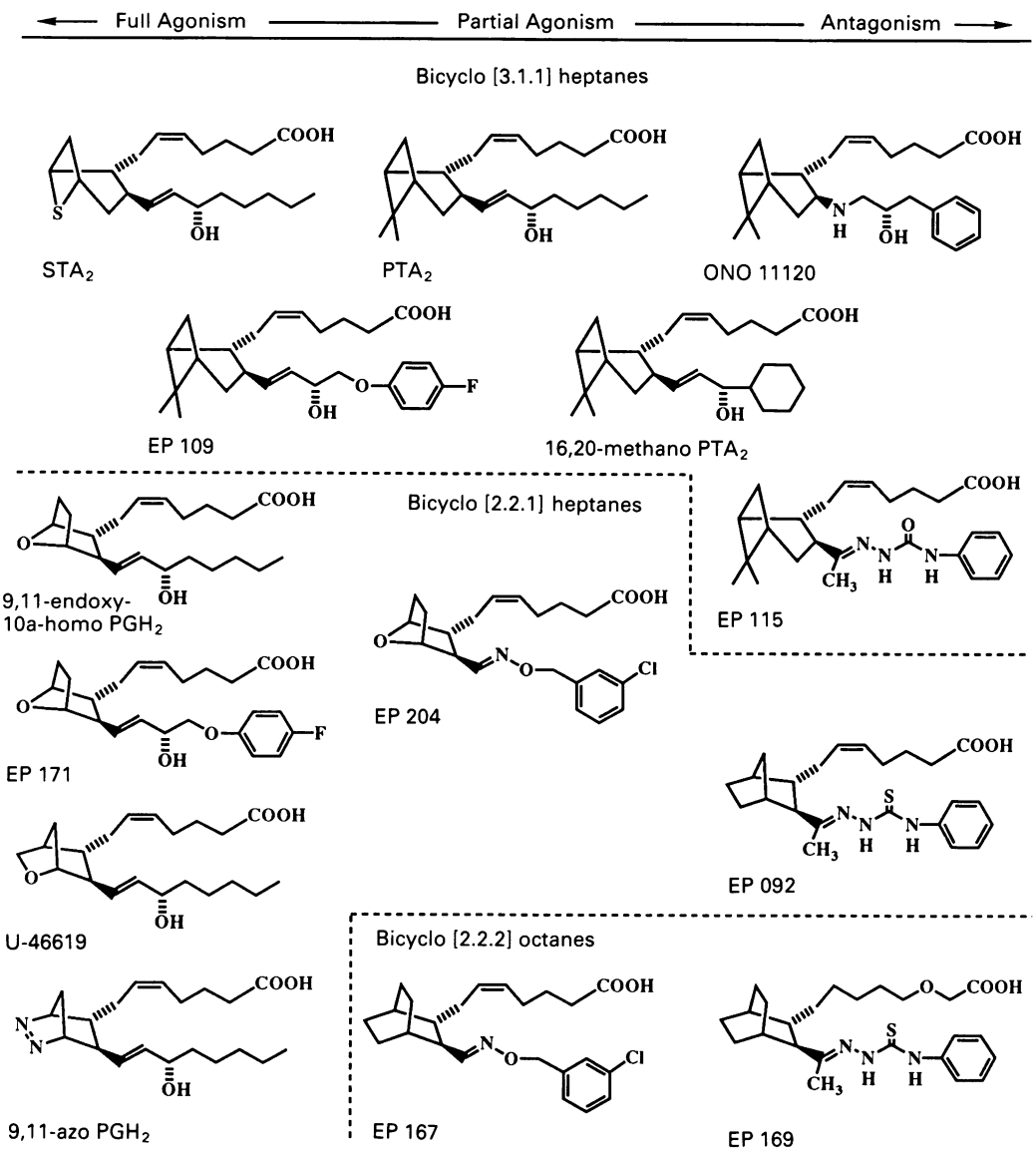

Figure 1 Synthetic analogues of thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) arranged according to their ring structures and whether they are TP-receptor full agonists, partial agonists or antagonists. All compounds have the natural upper side chain with the exception of EP 169 and all have the side chains in a *trans* relationship to the ring system. U-46619, 9,11-azo PGH_2 and all the bicyclo[3.3.1]heptane analogues are chiral; the remaining compounds are racemic.

volunteers who had not taken non-steroidal anti-inflammatory drugs for at least 7 days previously, from the common carotid arteries of rabbits (2–4 kg) under pentobarbitone anaesthesia and from the abdominal aorta of rats (200–350 g) under ether anaesthesia. In a few experiments blood was obtained from human donors who had ingested 600 mg of aspirin 12 and 2 h before collection. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 200 *g* for 20 min. PGI_2 was added to the PRP (about 30 nM for human and 300 nM for rat and rabbit) and a platelet pellet prepared by centrifugation at 600 *g* for 20 min. The pellet was suspended in calcium-free Krebs solution (composition given below) at 37°C and the space above the suspension vented with 95% O_2/5% CO_2. At least 30 min was allowed for the PGI_2 to decay.

Platelet shape change and aggregation were measured with a Chrono-Log dual-channel aggregometer (Born, 1962). Each cuvette (siliconised glass) contained 0.4 ml washed platelet suspension, 0.02 ml 25 mM $CaCl_2$ solution and 0.06 ml 0.9% NaCl solution (in which inhibitors were dissolved). Stirring was achieved with a Teflon-coated magnetic follower revolving at 1000 r.p.m. Aggregating agents were added in 0.02 ml 0.9% NaCl solution.

To measure the relative potency of a full agonist in a single experiment, concentration-aggregation relationships for the standard agonist (U-46619) followed by the test agonist were obtained on one channel of the aggregometer, with duplicate measurements being made on the second channel, test agonist before standard. Each aggregation response was measured as the maximum pen deflection occurring within 1 min of agonist addition and was expressed as a percentage of the U-46619 maximum. Log concentration-response curves (log CRCs) were plotted using the mean data and an equi-effective molar ratio (EMR) calculated at the 50% level. In the case of EP 171, aggregation waves had slower time courses than those of other agonists and responses were measured at 2 min after drug addition (see Jones *et al.*, 1989).

For partial agonists (and EP 171) shape change responses were measured using a similar protocol. Large shape change responses were often curtailed by the onset of primary aggregation and the resulting log concentration-response curves were bell-shaped. The effect was most marked with U-46619 and EP 109, their apparent maxima being slightly less than those of EP 167 and EP 204. In each experiment the EC_{50} value for each agonist was measured at the 50% maximum shape change level of the agonist producing the highest

maximum. This means that shape change EC_{50} values presented here are likely to be smaller than 'true values' from experiments in which aggregation was specifically suppressed. However, potency ranking is unlikely to be drastically affected by this limitation. The partial agonists were also tested for their ability to inhibit maximal aggregation induced by U-46619 (300 nM) and either platelet-activating factor (PAF, 100 and 10 nM on human and rabbit platelets) or adenosine 5'-diphosphate (ADP, 1 μM on rat platelets), the cyclo-oxygenase inhibitor flurbiprofen (1 μM) being present in the latter two tests. Each compound was added with minimal agitation to the platelet suspension at room temperature and 15 s later the cuvette was placed in the heated cell block of the aggregometer and normal stirring started. After 2 min the aggregating agent was added and the extent of aggregation at 1 min recorded as described previously. With this procedure the partial agonist alone induced shape change but no detectable aggregation. Log concentration-inhibition curves were constructed and IC_{50} values calculated. EP 109 was not tested in this manner since aggregation usually occurred as the cuvette contents heated up.

Antagonists were usually incubated at 37°C for 2 min before agonist addition and aggregation responses were measured at 1 min as above. In each experiment a control CRC for U-46619 followed by a CRC for U-46619 in the presence of a concentration of antagonist giving a dose-ratio of about 5 were obtained on one channel. On the second channel a CRC in the presence of an antagonist concentration some 3 to 4 times higher (intended to give a dose ratio not greater than 20) followed by a control CRC were obtained. In successive experiments with any one antagonist the order of the low and high antagonist treatments was reversed. Dose-ratios were calculated from log CRCs at the 50% maximum aggregation level. pA_2 values (calculated as the sum of log (dose ratio − 1 and −log [antagonist]) for the two antagonist concentrations were averaged, since they did not differ by more than 0.2 log units. In the case of EP 169 and AH 23848, maximum block was not achieved within 2 min and incubation periods of 5 and 10 min respectively were used. Aggregation waves were also slower at concentrations of antagonist producing dose-ratios in excess of 5; all responses were therefore measured at 2 min after agonist addition.

Smooth muscle preparations

Thoracic aortae were obtained from male rats and rabbits and tracheae from male and female guinea-pigs killed by stunning and exsanguination. Rings of tissue (3 mm wide) were suspended between stainless steel hooks in 10 ml organ baths. The Krebs bathing solution (NaCl 118, KCl 5.4, $MgSO_4$ 1.0, $CaCl_2$ 2.5, NaH_2PO_4 1.1, $NaHCO_3$ 25, dextrose 10 mmol l^{-1}) was gassed with 95% O_2/5% CO_2 and maintained at 37°C. Tension was recorded with Grass FT03 force-displacement transducers linked to a Grass polygraph. The spontaneous tone of the guinea-pig trachea was abolished by inclusion of 1 μM indomethacin and 20 nM atropine sulphate in the bathing solution.

Cumulative concentration-response relationships were obtained for the agonists and partial agonists, with U-46619 used as the standard agonist. With antagonists, the preparation was exposed to two series of cumulative doses of U-46619, followed by exposure to the antagonist (50 min) and then a further series of U-46619 doses. Dose-ratios at three antagonist concentrations (1:5:25) from 3 or 4 preparations were obtained. pA_2 values were calculated for individual dose-ratios and the regression of pA_2 on log [antagonist] determined. If the regression coefficient, r, is not statistically significant from zero ($P = 0.05$), then the Schild equation for competitive antagonism, log (dose-ratio − 1) versus log [antagonist] (Arunlakshana & Schild, 1959), is obeyed and the best estimate of pA_2 is the mean value (Mackay, 1978). Slopes of best fit for the Schild plots were also determined.

Compounds

The following prostanoids were prepared in our laboratory and essential details of the synthetic methods are to be found in Wilson *et al.* (1982, 1988): *rac* 10a-homo-15S-hydroxy-9α, 11α-epoxy-prosta-5Z,13E-dienoic acid (9,11-endoxy-10a-homo PGH_2) and its 16*p*-fluorophenoxy-ω-tetranor (EP 171) and 13-(*m*-chlorobenzyloxyimino)-ω-heptanor (EP 204) analogues; *rac* 9α,11α-ethano-ω-heptanor-13-methyl-13-phenyl-thio-carbamoyl-hydrazino-prosta-5Z-enoic acid (EP 092); 11a-carba-15S-hydroxy-9α,11α-isopropylideno-prosta-5Z,13E-dienoic acid (PTA_2) and its 16*p*-fluorophenoxy-ω-tetranor (EP 109), ω-heptanor-13-methyl-13-phenylecarbamoylhydrazino (EP 115) and 16,20-methano (16,20-methano PTA_2) analogues; 9α,11α-ethano-ω-heptanor-10a-homo-13-(*m*-chlorobenzyl-oxyimino)prosta-5Z-enoic acid (EP 167) and 4a,10a-dihomo-9α,11α-ethano-ω-heptanor-3-oxa-13-phenyl-thio-carbamoyl-hydrazino-prostanoic acid (EP 169); 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$. PGH_2 was prepared from $PGF_{2\alpha}$ by the method of Porter *et al.* (1979).

The following compounds were gifts and are gratefully acknowledged: 9α,11α-thia-11a-carba-prosta-5Z,13E-dienoic acid (STA_2) and 11a-carba-12-(2'S-hydroxy-3'-phenylpropylamino)-9α,11α-isopropylideno-ω-octanor-prost-5Z-enoic acid (ONO 11120) from ONO, Japan; *p*-(2-benzenesulphonamidoethyl)-phenoxyacetic acid (BM 13177) from Boehringer-Mannheim, W. Germany; *rac* 9α-(biphenylyl)methoxy-12β-(N-morpholinyl)-ω-octanor-11-oxo-prost-4Z-enoic acid (AH 23848), 9α-(biphenylyl)methoxy-11β-hydroxy-12β-(N-piperidinyl)-ω-octanorprost-4Z-enoic acid (GR 32191) and 6-isopropoxy-9-oxoxanthen-2-carboxylic acid (AH 6809) from Glaxo, UK; *rac* 15S-hydroxy-9-oxo-16-phenoxy-ω-tetranorprost-13E-enoic acid (MB 28767) from Rhone-Poulenc, U.K.; *rac* 16-*p*-chlorophenoxy-ω-tetranor PGE_2 (ICI 80205) from ICI Pharmaceuticals, U.K.; PGI_2 from Schering AG, Berlin, W. Germany; dazoxiben from Pfizer, U.K.; flurbiprofen from Boots, U.K.

11α,9α-Epoxymethano-15S-hydroxy-prosta-5Z,13E-dienoic acid (U-46619), 9α,11α-azo-15S-hydroxy-prosta-5Z,13E-dienoic acid (9,11-azo PGH_2), 16,16-dimethyl PGE_2, 16,16-dimethyl $PGF_{2\alpha}$ and 15-methyl $PGF_{2\alpha}$ were purchased from Upjohn Diagnostics Ltd., U.S.A. Indomethacin, PAF and ADP were purchased from Sigma.

Prostanoids were usually dissolved in absolute ethanol to give concentrations of 5–20 mM and stored at −20°C. Aqueous stock solutions (100–500 μM) were prepared by adding an equimolar equivalent of NaOH to a portion of the ethanol solution, evaporating the ethanol with a nitrogen stream and dissolving the sodium salt residue in 0.9% NaCl solution. AH 23848, supplied as the calcium salt, was dissolved with gentle warming in 0.9% NaCl solution containing a trace of solid $NaHCO_3$. Aqueous stocks of EP 092, EP 115, EP 169 and AH 23848 were freshly prepared for each experiment.

Results

Washed platelet suspensions

U-46619 always produced complete and irreversible aggregation in washed platelet suspensions from man, rat and rabbit and was used as the standard agonist in agonist and antagonist potency determinations. Mean EC_{50} values for shape change on human, rat and rabbit platelets were 4.8 (range 4.1–6.5), 6.0 (3.9–8.7) and 7.3 (5.3–11) nM respectively (n = 24–26) and for aggregation 82 (55–130), 145 (108–310) and 65 (42–120) nM respectively (n = 96–108).

Full agonists In addition to U-46619, seven other prostanoids behaved as full agonists (see Figure 1 for structures) and the same order of potency was found on the three platelet preparations: EP 171 > STA_2 > 9,11-azo PGH_2 >

Table 1 Potencies of TP-receptor full agonists on human, rat and rabbit platelets

Agonist	*Platelet aggregation: equi-effective molar ratios*		
	Man	*Rat*	*Rabbit*
EP 171	0.044 ± 0.007	0.032 ± 0.007	0.045 ± 0.005
	(0.025 ± 0.005)*	(0.022 ± 0.005)*	(0.026 ± 0.004)*
STA_2	0.30 ± 0.05	0.19 ± 0.04	0.21 ± 0.03
9,11-Azo PGH_2	0.46 ± 0.06	0.50 ± 0.04	0.30 ± 0.06
9,11-Endoxy-10a-homo PGH_2	0.97 ± 0.05	0.99 ± 0.07	0.52 ± 0.04
U-46619	1.00	1.00	1.00
PGH_2 §	1.8 ± 0.1	1.5 ± 0.1	1.3 ± 0.1
16-*p*-Fluorophenoxy ω-tetranor $PGF_{2\alpha}$	3.2 ± 0.2	1.1 ± 0.1	1.4 ± 0.2
16,16-Dimethyl $PGF_{2\alpha}$	7.3 ± 0.6	8.2 ± 0.8	12.5 ± 2.1

Values are means (± s.e.mean) for 4 determinations.
* Equi-effective molar ratios for shape change.
§ Dazoxiben 80 μM present.

9,11-endoxy-10a-homo PGH_2 > U-46619 > PGH_2 = 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ > 16,16-dimethyl $PGF_{2\alpha}$. The activity of PGH_2 was measured in the presence of 80 μM dazoxiben (Randall *et al.*, 1981) to prevent its conversion to TXA_2 by platelet thromboxane synthetase. In individual experiments, log concentration-aggregation curves for test agonists were parallel to that of U-46619. Mean equi-effective molar ratios (EMR) derived from 4 separate experiments are given in Table 1. In view of the difficulty of quantifying the slow aggregation waves produced by EP 171, shape change responses were used to obtain an additional potency comparison (Table 1); EP 171 was about 40 times more potent than U-46619 on each platelet preparation.

On human platelets, increasing the concentration of STA_2 above that required for maximal aggregation (100 nM) led to a reduced response (Figure 2); a similar depression of the maximum response was not seen on rat or rabbit platelets. This effect was studied further in platelet suspensions pretreated with a high concentration (6 μM) of the TP-receptor antagonist EP 092; at this concentration the antagonism is unsurmountable (U-46619 dose-ratio > 200). On human platelets STA_2 inhibited aggregation induced by PAF (100 nM) over the concentration-range corresponding to the depression of its own maximum when acting alone (Figure 2). In contrast, STA_2 (up to 3 μM) had little inhibitory effect (< 10%) on PAF (10 nM)-induced aggregation in rabbit platelets (n = 4) and on ADP (1 μM)-induced aggregation in rat platelets (which are insensitive to PAF) (n = 4). Under similar conditions U-46619 (up to 3 μM) did not affect PAF action on human platlets.

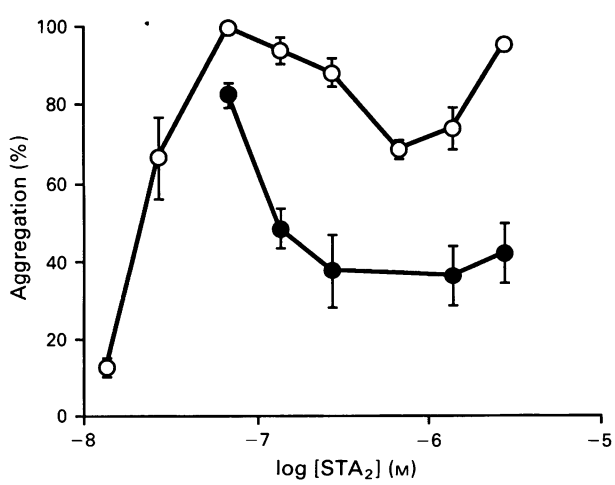

Figure 2 Washed human platelets: log concentration-response curves for STA_2 as an aggregating agent (○) and as an inhibitor of aggregation induced by platelet-activating factor (PAF) (100 nM) (●). The TP-receptor antagonist EP 092 (6 μM) was present in the PAF experiments. Each value is the mean from 4 experiments; vertical bars show s.e.mean.

Partial agonists Five prostanoids, EP 109, EP 167, EP 204, PTA_2 and 16,20-methano PTA_2 behaved in general as partial agonists, eliciting only shape change (EC_{50} values given in Table 2) or shape change and reversible aggregation (relative maxima in Table 2). Similar profiles were observed in platelet suspensions from aspirin-treated human donors. Of the three bicyclo[3:1.1]heptane analogues, EP 109 had the strongest agonist activity. It induced maximal shape change in all three platelet systems and was about twice as potent as U-46619. On rabbit platelets, EP 109 was also about twice as active as U-46619 with respect to aggregation and can be classed as a full agonist. Although EP 109 produced irreversible aggregation on some samples of human and rat platelets, it always had a shallower log CRC than U-46619 in these experiments; on other platelet samples EP 109 could only induce reversible aggregation waves (maximum concentration tested = 2 μM).

EP 167, EP 204 and PTA_2 inhibited irreversible aggregation induced by U-46619 with IC_{50} values respectively of 0.64 ± 0.08, 0.94 ± 0.06 and 0.10 ± 0.09 μM on human platelets, 0.55 ± 0.07, 1.11 ± 0.10 and 1.25 ± 0.05 μM on rat platelets and 0.95 ± 0.03, 0.98 ± 0.05 and 9.8 ± 1.5 μM on rabbit platelets. With the exception of PTA_2 on human platelets, these inhibitory effects were specific for U-46619, aggregation responses to PAF on human and rabbit platelets (100 and 10 nM respectively) and to ADP (1 μM) on rat platelets being unaffected (EP analogues at 1 μM and PTA_2 at 1–20 μM). PTA_2 at 50–500 nM markedly inhibited PAF-induced aggregation in human platelets.

16,20-methano PTA_2 (0.5–5 μM) elicited small shape change responses in each of the three platelet systems. Since the maximum effects were never greater than 50% of the U-46619 maxima it was decided to treat the compound as an antagonist (see later).

Antagonists Five of the seven antagonists studied, EP 092, ONO 11120, 16,20-methano PTA_2, EP 115 and BM 13177 inhibited aggregation induced by U-46619 without changing the nature of aggregation waves. In individual experiments parallel shifts in the U-46619 log concentration-response curve were obtained. Mean pA_2 values from 4 or 5 experiments together with ranges are given in Table 3. It can be seen that antagonist potencies are very similar on human and rat platelets and, with the exception of BM 13177, considerably lower on rabbit platelets.

Surmountable block of U-46619-induced aggregation, with parallel displacement of log concentration-response curves was also observed for the other two antagonists, EP 169 and AH 23848, on rabbit platelets. However these two compounds produced an unsurmountable block on both human (Figure 3) and rat platelets. In order to obtain at least some estimate of blocking potency, a pA_2 value was calculated for the lowest concentrations of EP 169 (10 nM) and AH 23848 (15 nM), where little change in the U-46619 aggregation profile was seen and parallel shifts of the U-46619 log concentration-response curves were obtained. These values are denoted by

Table 2 Potencies of TP-receptor partial agonists on human, rat and rabbit platelets and on guinea-pig trachea, rat aorta and rabbit aorta

	EC_{50} *for shape change* (nM) *and relative aggregation maximum*			EC_{50} *for contraction* (nM) *and relative maximum*		
Prostanoid	*Human platelets*	*Rat platelets*	*Rabbit platelets*	*Guinea-pig trachea*	*Rat aorta*	*Rabbit aorta*
U-46619 (full agonist)	4.8 ± 0.2 (100%)	5.5 ± 0.5 (100%)	7.2 ± 0.5 (100%)	7.8 ± 1.0 (100%)	20.2 ± 3.1 (100%)	12.3 ± 3.8 (100%)
EP 109	1.6 ± 0.4 (62–95%)	3.7 ± 0.6 (10–100%)	3.1 ± 0.5 (100%)	2.6 ± 0.9 (46–70%)	8.3 ± 0.7 (38–63%)	4.4 ± 1.2 (75–94%)
EP 167	21 ± 6 (3–22%)	33 ± 6 (12–16%)	76 ± 31 (5–47%)	57 ± 10 (62–78%)	80 ± 18 (55–59%)	61 ± 5 (58–66%)
EP 204	47 ± 7 (4–11%)	210 ± 17 (10–14%)	96 ± 23 (5–34%)	104 ± 25 (84–88%)	590 ± 270 (50–61%)	119 ± 6 (65–76%)
PTA_2	145 ± 43 (0%)	170 ± 55 (0%)	290 ± 44 (0–3%)	~500 (4–13%)	~400 (4–16%)	172 ± 33 (47–63%)
16,20-methano PTA_2	>5000* (0%)	>5000* (0%)	>5000* (0%)	—* (0%)	—* (0%)	640 ± 170 (22–42%)

EC_{50} values are means (± s.e.mean) for 5 experiments; on smooth muscle preparations EC_{50} values relate to the compound's own maximum. Relative maxima are expressed as ranges.
* pA_2 estimated by the Schild method (see Tables 3 and 4).

an asterisk in Table 3 to indicate that we have little, if any, evidence for reversible competitive antagonism by these two compounds.

The previously unreported compounds, 16,20-methano PTA_2, EP 115 and EP 169, each at 10 μM, did not inhibit PAF-induced aggregation of human and rabbit platelets nor ADP-induced aggregation of rat platelets.

Isolated smooth muscle preparations

Full agonists The potencies of a number of analogues on guinea-pig trachea, rat aorta and rabbit aorta were estimated in order to supplement published data. On guinea-pig trachea (5 μM AH 6809 present to block EP_1-receptors), rat aorta and rabbit aorta, mean EMR (U-46619 = 1.0, $n = 4$) were as follows: 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ 1.2, 1.2, 1.6; MB 28767 4.7, 5.9, 3.0; 16,16-dimethyl PGE_2 (no value), 54, 30; 15-methyl $PGF_{2\alpha}$ 177, 180, 155.

Partial agonists EP 109, EP 167, EP 204 and PTA_2 behaved as partial agonists on guinea-pig trachea, rat aorta and rabbit aorta. EC_{50} values and relative maxima are given in Table 2. Established responses to each prostanoid of about 80% of its own maximum were abolished within 20 min of addition of 1 μM EP 092. 16,20-methano PTA_2 (0.1–10 μM) elicited no contractile activity on guinea-pig trachea and rat aorta and was treated as a pure antagonist (see next section). However on the rabbit aorta this compound consistently behaved as a partial agonist (Table 2).

Table 3 Potencies of TP-receptor antagonists on human, rat and rabbit platelets

	pA_2		
Antagonist	*Human*	*Rat*	*Rabbit*
EP 169	8.30* (8.22–8.37)	8.48* (8.32–8.61)	7.24 (7.20–7.28)
AH 23848	8.05* (7.92–8.33)	8.19* (8.02–8.39)	6.88 (6.86–6.97)
EP 092	7.73 (7.31–7.95)	7.80 (7.65–7.99)	6.71 (6.67–6.85)
ONO 11120	7.49 (7.41–7.57)	7.38 (7.35–7.81)	5.95 (5.93–5.96)
EP 115	6.98 (6.94–7.03)	7.00 (6.96–7.05)	6.15 (6.07–6.25)
16,20-methano-PTA_2	7.24 (7.17–7.35)	6.85 (6.77–6.95)	5.70 (5.66–5.78)
BM 13177	6.21 (6.15–6.27)	6.11 (6.08–6.11)	5.87 (5.83–5.91)

Values are means and ranges for 5 experiments on human and 4 experiments on rat and rabbit platelets. The agonist was U-46619.
* Indicates that the pA_2 is calculated for a single low concentration of antagonist at which block is surmountable; higher concentrations produce unsurmountable block.

Antagonists On individual preparations of guinea-pig trachea, rat aorta and rabbit aorta, six antagonists, EP 169, AH 23848, EP 092, ONO 11120, 16,20-methano PTA_2 and BM 13177, produced parallel shifts of the log concentration-

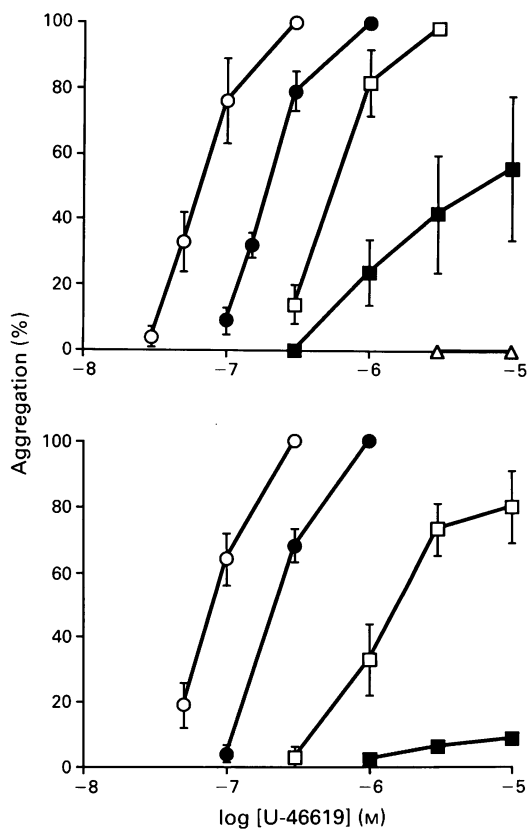

Figure 3 Unsurmountable antagonism of U-46619-induced aggregation in washed human platelets. In (a) the antagonist is EP 169 at 0 (○), 10 (●), 20 (□), 40 (■) and 200 (△) nM; in (b) the antagonist is AH 23848 at 0 (○), 15 (●), 30 (□) and 75 (■) nM. Each value is the mean of 4 experiments; vertical bars show s.e.mean.

Table 4 Potencies of TP-receptor antagonists on guinea-pig trachea, rat aorta and rabbit aorta

	Guinea-pig trachea		*Rat aorta*		*Rabbit aorta*	
Antagonist	pA_2	*Slope of Schild plot*	pA_2	*Slope of Schild plot*	pA_2	*Slope of Schild plot*
EP 169	8.77	1.00 ± 0.17	8.73	1.00 ± 0.17	7.16	1.27 ± 0.30
AH 23848	9.76	1.12 ± 0.20	8.47	0.96 ± 0.14	7.30	0.97 ± 0.17
GR 32191	9.43	0.97 ± 0.19	8.41	1.00 ± 0.20	—	—
EP 092	8.02	1.05 ± 0.12	8.55	0.95 ± 0.20	7.48	1.06 ± 0.14
ONO 11120	8.07	0.99 ± 0.21	7.14	0.89 ± 0.13	5.96	1.08 ± 0.19
16,20-methano PTA_2	7.14	1.01 ± 0.22	7.83	1.01 ± 0.15	partial agonist ($-\log EC_{50} = 6.2$)	
BM 13177	6.30	0.89 ± 0.17	6.25	1.12 ± 0.14	6.24	0.94 ± 0.11

U-46619 was used as the agonist.
The slope of the Schild plot is that of the least squares regression line (±95% confidence limits).

response curve to U-46619 with no suppression of the maximum response. For each antagonist/preparation no significant regression ($P > 0.05$) of pA_2 on log [antagonist] was found, indicating that the slope of the Schild plot did not differ from unity; pA_2 values and slopes of best fit (±95% confidence limits) for the Schild plot are given in Table 4. With the exception of BM 13177, pA_2 values were higher on the guinea-pig trachea and rat aorta than the rabbit aorta.

In view of the high potency of AH 23848 on guinea-pig trachea we decided to measure the potency of the chemically similar antagonist, GR 32191. Again very high TP-receptor antagonist potency was found on guinea-pig trachea (pA_2 = 9.43, Table 4). Our attempts to measure the potency of GR 32191 on rabbit aorta were thwarted by the complete insensitivity of this preparation to TP-receptor agonists over a period of about 6 months. It is of interest that jugular vein preparations from the same rabbits were highly responsive to TP-receptor agonists (U-46619 EC_{50} = 3 nM): GR 32191 block was surmountable and a pA_2 value of 7.22 was obtained.

Correlation of potencies

Potencies (log EMR) for full agonists on both the platelet and smooth muscle preparations are compared in Figure 4. The data include our previously published results for the smooth muscle preparations used in this study and for the dog saphenous vein (Jones *et al.*, 1982; 1989). Linear regression analysis gave highly significant correlations for all the 21 possible pairs of data sets: $r = 0.90$–0.99, $P < 0.01$.

For antagonists used in this study, pA_2s on human and rat platelets were highly correlated ($r = 0.98$, $P < 0.001$) and rabbit and rat platelet values were also well correlated ($r = 0.89$, $P < 0.01$). pA_2 values for rabbit aorta *versus* guinea-pig trachea, or human platelets or rat platelets, rat aorta *versus* rabbit platelets, and guinea-pig trachea *versus* rabbit platelets were not correlated ($P > 0.05$). The remaining 8 pairs of data sets showed weak correlations ($r = 0.77$–0.91, $P < 0.05$).

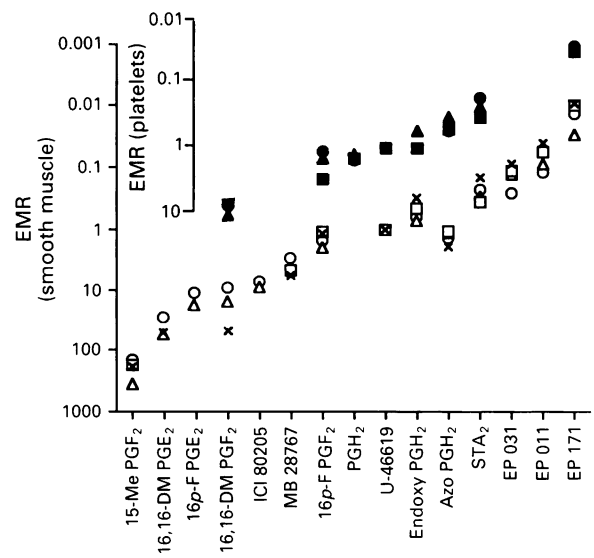

Figure 4 Comparison of TP-agonist potencies (equi-effective molar ratio, EMR, on log scale) for rabbit aorta (○), rat aorta (×), dog saphenous vein (△), guinea-pig trachea (□) and human (■), rat (●) and rabbit (▲) platelets. The platelet data have been displaced upwards for the sake of clarity.

Discussion

We contend that our results for full and partial agonist potencies on both the platelet and smooth muscle preparations (Jones *et al.*, 1982, 1989; these studies) provide no real evidence for the existence of TP-receptor subtypes.

With respect to the full agonists, both absolute and relative potencies are well correlated, particularly on the smooth muscle preparations where the EC_{50} values of the least (15-methyl $PGF_{2\alpha}$) and most potent (EP 171, Jones *et al.*, 1989) prostanoids differ by four orders of magnitude. Fewer agonists were tested on the platelet preparations, since some of the PGE analogues (ICI 80205, 16,16-dimethyl PGE_2 and MB 28767) with medium agonist potency at TP-receptors also potentiate both primary and secondary aggregation by an agonist action at a different prostanoid receptor (Jones & Wilson, 1990) (see Shio & Ramwell, 1972; Shio *et al.*, 1972 for the activity of PGE_2 in this respect).

STA_2 was found to have EC_{50} values for aggregation in the 15–30 nM range on human, rat and rabbit washed platelets and to be 3–5 times more potent than U-46619. These potencies agree well with data obtained on smooth muscle (Mais *et al.*, 1985; Toda *et al.*, 1986; Jones *et al.*, 1989) and thus there appear to be no major discrepancies in the agonist profile of STA_2. The only additional action (inhibition of PAF-induced aggregation in the presence of flurbiprofen) which might reduce its TP-agonist potency on platelets was found on human platelets and not rat or rabbit platelets. We believe this action is unlikely to reduce greatly the TP agonist potency of STA_2 on human platelets in view of the relative positions of the aggregatory and inhibitory log CRCs (Figure 2). However, if STA_2 were to be used as an agonist in antagonist studies on human platelets, its additional inhibitory action at the higher concentrations required to overcome the TP-receptor block could augment the block and the pA_2 would then be in error; U-46619 is clearly a more suitable agonist for this purpose. It is of interest that three TXA_2 analogues, CTA_2 and PTA_2 (Armstrong *et al.*, 1985) and STA_2 should show non-TP-receptor-mediated inhibitory actions on human platelets.

Turning to the stimulant actions of the partial agonists examined, there is also a close correspondence of the potency parameters (EC_{50} and relative maximum) of individual compounds on the six preparations investigated. On human platelets, irreversible inhibition of cyclo-oxygenase with aspirin did not significantly alter the partial agonist profiles. This is compatible with our previous experiments with the cyclo-oxygenase flurbiprofen on human PRP, where we showed that only a minor part of the irreversible aggregation and associated release reaction induced by U-46619 is due to endogenous prostaglandin endoperoxides and TXA_2 (Armstrong *et al.*, 1985). The novel analogues EP 167 and EP 204 differ from the usual prostanoids which exhibit TP-receptor agonist activity in not having a 15-hydroxyl group in the w-chain (Figure 1). In terms of inhibitory activity, EP 167 and EP 204 specifically blocked U-46619-induced aggregation on human, rat and rabbit platelets, with similar IC_{50} values. We have already mentioned the non-TP receptor inhibitory activity of PTA_2 on human platelets; this must preclude the use of its IC_{50} value from our comparisons. On rat and rabbit platelets however the inhibitory action of PTA_2 appears to be specific for TP-receptors and its greater inhibitory potency against U-46619 on rat as opposed to rabbit platelets would appear to reflect a genuine difference in its affinity for the respective TP-receptors.

The strongest evidence however for TP-receptor heterogeneity comes from our pA_2 measurements for TP antagonists. Of the 15 possible pairs of data sets, only human and rat platelets showed very close agreement of affinities. Although there was significant correlation of pA_2 values for rabbit platelets versus human or rat platelets, absolute values, with the exception of BM 13177, were lower on rabbit platelets. It is of interest that Swayne *et al.* (1988) have reported a good correlation between the potencies of nine TP-receptor antagonists on human and rabbit platelets and have used this result to postulate homogeneity of TP-receptors. However, although a highly significant correlation ($P < 0.01$ using a multiple linear correlation matrix) was obtained, actual IC_{50} values for inhibition of U-46619-induced aggregation were always much higher (13–600 fold!) on rabbit platelets than on human platelets. There is obviously a danger of using statistical correlation in this way to decide on the identity of receptors. It is almost certain that plasma protein binding contributes to these differences since the rabbit platelets were suspended in native plasma whereas the human platelets were in a buffer solution. It is difficult to make use of the Swayne data as the antagonist potencies are unlikely to be offset by a constant amount due to plasma protein binding.

Correlations between antagonist pA_2 values for all other pairs of preparations were found to be weak or not significant and the pA_2 values for some antagonists varied by 2 log units or more. Examination of the literature shows that other groups have also found widely differing affinities for TP antagonists on different preparations. On smooth muscle, pA_2 values for GR 32191 on 8 preparations varied between 7.14 and 8.72 (a range of 1.58 log units) (Lumley *et al.*, 1989); for the non-prostanoid TP-receptor antagonist SK&F 88046 on 4 preparations from 6.07 to 8.84 (2.77 log units) (Ohlstein *et al.*, 1988); for ICI 192605 on 6 preparations from 8.0 to 9.6 (1.6 log units) (Jessup *et al.*, 1988). In relation to comparing TP-receptors in smooth muscle and platelets, Mais *et al.* (1985) also demonstrated poor agreement of pA_2 values for 12 analogues of ONO 11120 on human washed platelets and dog saphenous vein (e.g. 8.74 and 6.78 respectively for the 16,17-thia analogue of ONO 11120). These authors state that 'the current evidence strongly supports the notion that the TXA_2/PGH_2 receptor in platelets is distinct from that in blood vessels'. We believe that a generalisation of this nature is of no value, since the differences in pA_2 values within each tissue category are themselves large enough to indicate TP-receptor heterogeneity.

An alternative explanation for these antagonist affinity differences is that of differential access to the receptor biophase. This concept is easier to apply to smooth muscle preparations than platelets, where the densely packed cell mass of the former may contain physical barriers or concentrating processes which could drastically reduced ligand concentrations in the extracellular fluid. There are however considerable similarities between the properties of the TP agonists, whose actions suggest receptor homogeneity, and the TP antagonists where the opposite is true. For example, the affinity of the most potent agonist EP 171 (Jones *et al.*, 1989) is of the same order as the more potent antagonists. All the ligands are of similar bulk (Figure 1) and cover a similar range of lipophilicity. For example STA_2 and EP 167 are highly lipophilic whereas 16,16-dimethyl $PGF_{2\alpha}$ is hydrophilic; EP 169 is lipophilic whereas BM 13177 is hydrophilic. The only obvious difference is that three of the antagonists, AH 23848 (Brittain *et al.*, 1985), GR 32191 and ONO 11120, will exist mainly as zwitterions in neutral aqueous solution. It seems more likely that small variations in the accessory binding sites for antagonists (say the substitution of a single amino acid) could account for the affinity differences: these structural differences would have minimal effect on the potencies of the full and partial agonists. Leff & Martin (1986) have discussed similar propositions in relation to the characterisation of 5-HT_2-like receptors and have raised the question as to whether antagonists chemically related to the natural agonist are of more use in defining subtypes as opposed to antagonists which bear little chemical relation. It is of interest that in our studies, the TP-receptor antagonist which is least prostanoid-like in structure, BM 13177 (Patscheke & Stegmeier, 1984), shows least variation in antagonist affinity. At present it would seem prudent not to subclassify the TP-receptor (TP_1-, TP_2-, etc.) on the basis of the rather limited antagonist potency data available.

Finally we wish to comment on the different antagonist profile of EP 169 and AH 23848 on human and rat platelets compared to rabbit platelets. It is possible that the apparently non-competitive nature of the block on human and rat platelets can be accounted for in terms of genuine reversible competition between agonist and antagonist, if agonist occupancy is incomplete at the time of response measurement due to slow dissociation of the antagonist. We have previously postulated that a similar mechanism could account for the slow irreversible aggregation waves to U-46619 seen on human platelets after treatment with high concentrations of EP 092 (Armstrong *et al.*, 1985). EP 169 and AH 23848 are more potent than EP 092 and they may well have smaller dissociation rate constants (k_2) than EP 092. Ligand binding studies indicate that potent TP antagonists can have half-lives for dissociation from TP-receptors compatible with our hypothesis. Armstrong *et al.* (1989) have shown that GR 32191 has a dissociation $t_{1/2}$ of at least 8 min in human washed platelets. GR 32191 also produces a marked slowing of U-46619 aggregation on human platelets, at concentrations slightly lower than those of AH 23848 used in the present study (Lumley *et al.*, 1989). These workers allowed up to 20 min for the agonist to overcome the block and analysed the results by the Schild procedure ($pA_2 = 8.78$, slope = 1.31). However, it is our experience of working with washed platelets that prolonged stirring alone can often lead to spontaneous aggregation and therefore one can never be entirely sure that the apparent surmountability of the block is entirely due to the dissociation of antagonist from TP-receptors. Clearly there are major difficulties in accurately estimating the pA_2 values of potent TP-receptor antagonists on platelet preparations using aggregation as the response.

References

ANDERSON, L. & MACINTYRE, D.E. (1982). Differences between thromboxane A_2 receptors on rabbit and human platelets. *Br. J. Pharmacol.*, **77**, 546P.

ARMSTRONG, R.A., JONES, R.L., PEESAPATI, V., WILL, S.G. & WILSON, N.H. (1985). Competitive antagonism at thromboxane receptors in human platelets. *Br. J. Pharmacol.*, **84**, 595–607.

ARMSTRONG, R.A., LUMLEY, P. & HUMPHREY, P.P.A. (1989). Characteristics of [^{3}H] GR 32191 binding to the thromboxane (TP) receptor of human platelets. *Br. J. Pharmacol.*, **98**, 843P.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonism. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BORN, G.R.V. (1962). Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, **194**, 927–929.

BRITTAIN, R.T., BOUTAL, L., CARTER, M.C., COLEMAN, R.A., COLLINGTON, E.W., GEISOW, H.P., HALLETT, P., HORNBY, E.J., HUMPHREY, P.P.A., JACK, D., KENNEDY, I., LUMLEY, P., MCCABE, P.J., SKIDMORE, I.F., THOMAS, M. & WALLIS, C.J. (1985). AH 23848: a thromboxane receptor-blocking drug that can clarify the pathophysiological role of thromboxane A_2. *Circulation*, **72**, 1208–1218.

JESSUP, C.L., JESSUP, R. & WAYNE, M. (1988). ICI 192605; a potent selective thromboxane A_2 receptor antagonist on smooth muscle. *Br. J. Pharmacol.*, **95**, 675P.

JONES, R.L., PEESAPATI, V. & WILSON, N.H. (1982). Antagonism of the thromboxane-sensitive systems of the rabbit aorta, dog saphenous vein and guinea-pig trachea. *Br. J. Pharmacol.*, **76**, 423–438.

JONES, R.L. & WILSON, N.H. (1990). An EP_3-receptor may mediate prostaglandin E-induced potentiation of aggregation in human platelets. *Br. J. Pharmacol.*, **101**, 522P.

JONES, R.L., WILSON, N.H. & LAWRENCE, R.A. (1989). EP 171: a high affinity thromboxane A_2 mimetic, the actions of which are slowly reversed by receptor blockade. *Br. J. Pharmacol.*, **96**, 875–887.

KATSURA, M., MIYAMOTO, T., HAMANAKA, N., KONDO, K., TERADA, T., OHGAKI, Y., KAWASAKI, A. & TSUBOSHIMA, M. (1983). *In vitro* and *in vivo* effects of new powerful thromboxane antagonists (3-alkylamino pinane derivatives). *Adv. Prostaglandin Thromboxane Leukotriene Res.*, **11**, 327–332.

LEFF, P. & MARTIN, G.R. (1986). Peripheral 5-HT_2-like receptors. Can they be classified with the available antagonists? *Br. J. Pharmacol.*, **88**, 585–593.

LUMLEY, P., WHITE, B.P. & HUMPHREY, P.P.A. (1989). GR 32191, a highly potent and specific thromboxane blocking drug on platelets and vascular and airways smooth muscle *in vitro*. *Br. J. Pharmacol.*, **97**, 783–794.

MACKAY, D. (1978). How should values of pA_2 and affinity constants for pharmacological competitive antagonists be calculated? *J. Pharm. Pharmacol.*, **30**, 312–313.

MAIS, D.E., SAUSSY, D.L., CHAIKHOUNI, A., KOCHEL, P.J., KNAPP, D.R., HAMANAKA, N. & HALUSHKA, P.V. (1985). Pharmacologic characterisation of human and canine thromboxane A_2/prostaglandin H_2 receptors in platelets and blood vessels: evidence for different receptors. *J. Pharmacol. Exp. Ther.*, **233**, 418–424.

NARUMIYA, S., OKUMA, M. & USHIKUBI, F. (1986). Binding of a radio-iodinated 13-azapinane thromboxane antagonist to platelets: correlation with antiaggregatory activity in different species. *Br. J. Pharmacol.*, **88**, 323–331.

OHLSTEIN, E.H., KOPIA, G.A., ZEID, R.L., VALOCIK, R.W., HOROHONICH, S., HIEBLE, J.P. & WASSERMAN, M.A. (1988). Effects of the thromboxane receptor antagonist SK&F 88046 in the canine, monkey and human coronary vasculature. *Prostaglandins*, **36**, 69–84.

PATSCHEKE, H. & STEGMEIER, K. (1984). Investigations on the selective nonprostanoic thromboxane antagonist BM 13177 in human platelets. *Thromb. Res.*, **33**, 277–288.

PORTER, N.A., BYERS, J.D., HOLDEN, K.M. & MENZYL, D.B. (1979). Synthesis of prostaglandin H_2. *J. Amer. Chem. Soc.*, **101**, 4319–4323.

RANDALL, M.J., PARRY, M.J., HAWKESWOOD, E., CROSS, P.E. & DICKINSON, R.P. (1981). UK-37248, a novel selective thromboxane synthetase inhibitor with anti-aggregatory and anti-thrombotic activity. *Thromb. Res.*, **23**, 145–162.

SHIO, H. & RAMWELL, P.W. (1972). Effect of prostaglandin E_2 and aspirin on the secondary aggregation of human platelets. *Nature, (New Biol.)*, **236**, 45–46.

SHIO, H., RAMWELL, P.W. & JESSUP, J. (1972). Prostaglandin E_2: Effects on aggregation, shape change and cAMP of rat platelets. *Prostaglandins*, **1**, 29–35.

SWAYNE, G.T.G., MAGUIRE, J., DOLAN, J., RAVAL, P., DANE, G., GREENER, M. & OWEN, D.A.A. (1988). Evidence for homogeneity of thromboxane A_2 receptor using structurally different antagonists. *Eur. J. Pharmacol.*, **152**, 311–319.

TODA, N., NAKAJIMA, M., OKAMURA, T. & MIYAZAKI, M. (1986). Interactions of thromboxane A_2 analogs and prostaglandins in isolated dog arteries. *J. Cardiovasc. Pharmacol.*, **8**, 818–825.

WILSON, N.H., JONES, R.L., MARR, C.G. & MUIR, G. (1988). Synthesis of oxabicyclo[2.2.1]heptane prostanoids having thromboxane-like activity at subnanomolar concentrations. *Eur. J. Med. Chem.*, **23**, 359–364.

WILSON, N.H., PEESAPATI, V., JONES, R.L. & HAMILTON, K. (1982). Synthesis of prostanoids with bicyclo[2.2.1]heptane, bicyclo[3.1.1]heptane, and bicyclo[2.2.2]octane ring systems. Activities of 15-hydroxy-epimers on human platelets. *J. Med. Chem.*, **25**, 495–500.

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