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Competitive antagonism at thromboxane receptors in human platelets

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1 The inhibitory effects of three prostanoid analogues, EP 045, EP 092 and pinane thromboxane A_2 (PTA_2), on the aggregation of human platelets *in vitro* have been investigated.

2 In diluted platelet-rich plasma (PRP), EP 045 (20 μM) and EP 092 (1 μM) completely inhibited irreversible aggregation responses to thromboxane A_2 (TXA_2), prostaglandin H_2 (PGH_2) and five chemically stable thromboxane mimetics, including 11,9-epoxymethano-PGH_2 and 9,11-azo-PGH_2. Reversible aggregation produced by the prostanoid analogue, CTA_2, was also inhibited. The block of the stable agonist action was surmountable. In plasma-free platelet suspensions EP 045 and EP 092 were more potent antagonists. Schild analysis indicated a competitive type of antagonism for EP 045 (affinity constant of $1.1 \times 10^7\ M^{-1}$); the nature of the EP 092 block is not clear.

3 Primary aggregation waves induced by ADP, platelet activating factor (Paf) and adrenaline were unaffected by EP 045 and EP 092, whereas the corresponding second phases of aggregation were suppressed. Aggregation and 5-hydroxytryptamine (5-HT) release induced by either PGH_2 or 11,9-epoxymethano-PGH_2 were inhibited in a parallel manner by EP 045. Inhibition of thromboxane biosynthesis is not involved in these effects.

4 EP 045 and EP 092 did not raise adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels in the platelet suspensions.

5 In plasma-free platelet suspensions PTA_2 produced a shape change response which could be blocked by EP 045. PTA_2, therefore, has a thromboxane-like agonist action. The block of the aggregatory action of 11,9-epoxymethano-PGH_2 by PTA_2 appears to be mainly due to competition at the thromboxane receptor. However, PTA_2 produced a slight rise in cyclic AMP levels; this could be due to a very weak stimulant action on either PGI_2 or PGD_2 receptors present in the human platelet. Functional antagonism by PTA_2 may therefore augment its thromboxane receptor blocking activity.

6 The results are discussed in terms of (a) the specificity of antagonism produced by EP 045, EP 092 and PTA_2, (b) the validity of affinity constant determinations for receptor antagonists when aggregation is the biological response, and (c) the characteristics of the human platelet thromboxane receptor in comparison with those of thromboxane receptors in smooth muscle.

Introduction

Addition of either the prostaglandin endoperoxides, prostaglandin G_2 (PGG_2) and PGH_2 (Hamberg *et al.*, 1974), or thromboxane A_2 (TXA_2) (Svensson *et al.*, 1976) to a stirred suspension of human platelets induces a rapid aggregation response. It is thought that these agents, by acting as agonists at receptors situated in the platelet plasma membrane, raise the concentration of free Ca^{2+} in the cytosol and hence produce a shape change and a primary aggregation wave (Pollock *et al.*, 1984). Aggregation may also be produced by causing the platelet to synthesize prostaglandin endoperoxides and TXA_2 via arachidonic acid released from phospholipid stores. These conversions occur in concert with the platelet release reaction and the PGG_2/PGH_2 and TXA_2 produced are major initiators of secondary irreversible aggregation (Smith *et al.*, 1974; Willis *et al.*, 1974; Malmsten *et al.*, 1975; Kinlough-Rathbone *et al.*, 1977).

Our recent studies have centred on the identification of compounds with a specific blocking action at thromboxane receptors. We have shown that one of our synthetic compounds, coded EP 045 (Figure 1), is a specific competitive thromboxane receptor antagonist on several smooth muscle preparations (Jones *et al.*, 1982). Preliminary investigation of EP 045 on human platelets revealed inhibitory activity consistent with

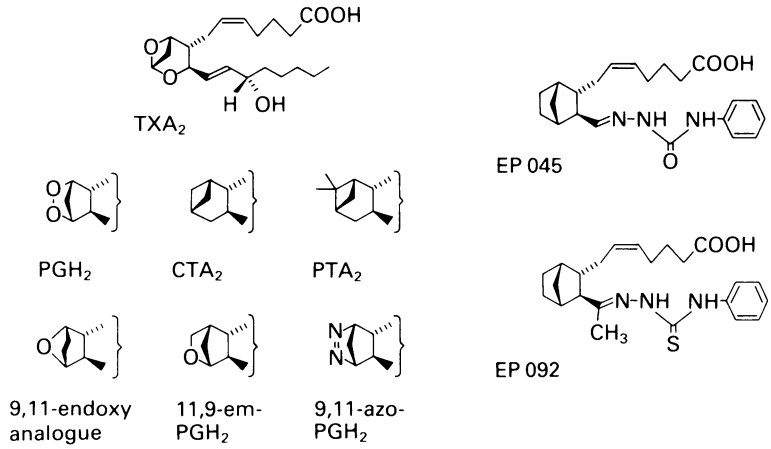

Figure 1 Structures of some of the prostanoids used in this study. The bracket indicates that the upper and lower chains are identical to those found in the natural 2-series prostanoids, e.g. thromboxane A_2 (TXA_2). The 9,11-endoxy analogue, EP 045 and EP 092 are racemic. The 11,9-epoxymethano (11,9-em) analogue of prostaglandin H_2 (PGH_2), 11,9-em-PGH_2, was used as the standard agonist.

thromboxane receptor blockade (Jones *et al.*, 1983). In this investigation we have examined the activity of EP 045 in greater detail with a view to answering the following questions: (a) Is EP 045 a specific competitive antagonist and if so; can we accurately measure by pharmacological means its affinity for the thromboxane receptor of the human platelet? (b) Are aggregation responses to TXA_2, PGH_2 and their synthetic analogues blocked to similar extents by EP 045 and is it likely that these agonists act at the same receptor on the human platelet? (c) How effective is EP 045 as an inhibitor of the platelet release reaction and second phase aggregation?

We have also compared EP 045 with a closely related analogue EP 092 (Figure 1). We have found the latter compound, owing to its greater blocking potency and longer duration of action, to be of considerable use in *in vivo* experiments, and we intend to use it in future studies.

Finally we have examined the actions of pinane thromboxane A_2 (PTA_2) which is claimed to be a thromboxane receptor antagonist on human platelets (Nicolaou *et al.*, 1979). PTA_2 is of particular interest to us, since we have consistently found it to be a partial agonist on thromboxane-sensitive smooth muscle preparations (Jones *et al.*, 1982; Dong & Jones, 1982), and we wished to know whether it was truly devoid of thromboxane-like agonist activity on the human platelet.

Our overall aim was to compare the pharmacological characteristics of the thromboxane receptor of the human platelet with those of thromboxane receptors in smooth muscle.

Methods

Preparation of platelet suspensions

All manipulations were carried out at room temperature. Blood (60 or 100 ml) was obtained from the median cephalic vein of human volunteers and clotting was prevented by addition of acid citrate-dextrose solution (1 ml per 5 ml of blood). Centrifugation at 180 *g* for 20 min afforded platelet-rich plasma (PRP). When a plasma-free suspension was required, PGI_2 (about 20 nM) was added to the PRP and centrifugation at 450 *g* for 20 min performed. The supernatant was discarded and the pellet was carefully suspended in Ca^{2+}-free Krebs solution. The platelet suspensions were kept under an atmosphere of 95% O_2/5% CO_2. The Krebs solution contained (mM): NaCl 118, KCl 4.7, $MgSO_4$ 0.57, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7.

We are grateful to P.J. Kerry for pointing out to us that the pH of the platelet suspension must be kept constant in order to prevent the fall in sensitivity to thromboxane-like action which occurs with time (Kerry & Paton, 1984).

Platelet aggregation measurements

Platelet aggregation was measured by the method of Born (1962), using a modified Cary 118C spectrophotometer. The cell block was maintained at 37°C and stirring was achieved with a stainless steel rod (60 mm long, 1.2 mm in diameter) revolving 1000 times per min. 'Diluted PRP' (i.e. 1.0 ml PRP, 1.0 ml

Krebs solution which had been gassed with 95% O_2/5% CO_2, and 0.4 ml 0.9% w/v NaCl solution (saline)) was placed in a 4 ml disposable polystyrene cuvette (LIP Ltd) and allowed 5 min to reach 37°C in a water bath. The cuvette was transferred to the cell block and 2 min later the aggregating agent was added in 0.1 ml. saline and the optical signal recorded for a further 2–5 min. EP 045, EP 092, PTA_2, dazoxiben, indomethacin and flurbiprofen were added 7 min before the aggregating agent, replacing part or all of the 0.4 ml saline; PGE_1, ZK 36374 and PGD_2 were added 2 min before the aggregating agent. In the plasma-free experiments the PRP was replaced by the suspension of platelets in Ca^{2+}-free Krebs solution.

In general, the aggregation response was taken as the maximum change in the optical signal occurring within the first 75 or 100 s after agonist addition (arbitrary units). For a primary wave proceeding into a secondary wave this means that the measured response virtually corresponds to the magnitude of the primary wave only.

5-Hydroxytryptamine (5-HT) release from human platelets

[^{14}C]-5-HT creatinine sulphate, specific activity 40 mCi mmol^{-1} (Amersham), was added to PRP (final concentration = 2.5 μM) and uptake was allowed to proceed for 30 min at 37°C. PRP (0.5 ml), Krebs solution (0.3 ml) and saline containing the potential inhibitor (0.2 ml) were placed in a silanized glass cuvette and allowed 2 min to equilibrate in a Bryson aggregometer. Stirring was achieved magnetically with a silanized steel bar. Two minutes after the addition of the aggregating agent to the cuvette duplicate 0.2 ml samples were withdrawn and each was added to 0.8 ml EDTA solution (0.4% EDTA in saline). After centrifugation (16,000 *g* for 30 s), 0.5 ml of the supernatant was added to 10 ml of scintillation fluid (PCS-Amersham-Searle/toluene, 2:1) and conventional scintillation counting carried out. The [^{14}C]-5-HT released was expressed as a percentage of the amount of [^{14}C]-5-HT initially taken up by the platelets.

Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) measurements

Cyclic AMP binding protein was purified from sheep flank muscle according to methods described by Miyamoto *et al.* (1969) and Gilman (1970). For use in the assay an aliquot of the stock protein suspension was diluted with assay buffer containing 0.1% bovine serum albumin. Each assay tube contained the following substances dissolved in 200 μl 0.05 M Tris-HCl buffer (pH 7.5): 0.80 μmol sodium EDTA, 0.9 pmol 8[^{3}H]-adenosine 3′:5′-cyclic phosphate (specific activity 30 Ci mmol^{-1}, Amersham), 0.013 mg binding protein, and a variable amount of 'cold' cyclic AMP (Sigma) or sample to be measured. The assay tubes were kept on ice for 2 h, then 100 μl of a suspension of charcoal absorbant was added, and the tubes centrifuged at 12,000 *g* for 2 min at 4°C. A 200 μl sample of the supernatant was withdrawn from each tube for conventional scintillation counting. In the absence of cold cyclic AMP, 62% of the added radioactivity was bound. The binding protein has the following cross-reactivities (cyclic AMP = 100%): cyclic GMP 3.9%, cyclic UMP 2.0%, ATP, ADP, AMP, GTP, GDP, GMP and cyclic 2′:3′-AMP < 0.002%.

The prostaglandin analogue under study was incubated with the platelet suspension (1 ml) at 37°C for 30 s. The reaction was quenched by addition of 2 ml ethanol and 5 min later the sample was centrifuged at 2000 *g*. The supernatant was removed and the pellet suspended in 1 ml ethanol:water (2:1). Centrifugation was repeated. The combined supernatants were evaporated to dryness at 55°C under a stream of nitrogen. The residue was dissolved in 0.5 ml assay buffer and centrifuged at 12,000 *g* for 30 min to remove insoluble material. Two 50 μl samples of the supernatant (diluted when necessary) were assayed. This extraction procedure gives a recovery for cyclic AMP of 87.3 ± 1.4% (s.e.mean, 16 replicates). Four measurements were made at each concentration level of the compound under study and a mean value calculated.

Measurement of thromboxane B_2 production by human platelets

Citrated PRP (2.5 ml) was incubated for 3 min at 37°C with 75 μg ml^{-1} palmitic acid. Arachidonic acid, final concentration 100 μg ml^{-1}, was then added and after 30 s the system was quenched by addition of 0.5 ml 2 M HCl (final pH ~ 3). The sample was extracted twice with equal volumes of ethyl acetate and the ethyl acetate evaporated to dryness. The residue was taken up first in 5 ml ethanol and 2.5 ml water, and then in 5 ml benzene. The phases were mixed and then centrifuged for a few minutes in a bench centrifuge to ensure a sharp interface between the layers. The upper benzene layer was discarded and the aqueous layer evaporated to dryness. The extraction with benzene removes most of the neutral lipids, fatty acids and mono-hydroxy fatty acids. The residue was dissolved in methanol and portions were processed for combined gas chromatography-mass spectrometry using a VG Micromass 70-70F spectrometer. The methyl ester-n-butyloxime-trimethylsilyl ether (Me-BuO-TMS) derivative was prepared by treatment with (a) diazomethane in diethyl ether for 5 min (b) O-n-butylhydroxylamine hydrochloride in pyridine (5 mg ml^{-1}) at 60°C for 1 h, (c) *N*,*N*-bis(trimethylsilyl)-tri-

fluoroacetamide (BSTFA) at 60°C for 15 min.

Two internal standards were used–11-deoxy-PGE_1 added before (a) above and the ethyl ester of TXB_2 added before (b) above. The gas chromatograph effluent was subjected to high resolution single ion detection: fragment ion intensities at m/e 280 (base peak of the 11-deoxy PGE_1 derivative) and m/e 301 (base peak of both the methyl and ethyl esters of TXB_2) were recorded continuously. The ratio of peak heights for m/e 301 (Me)/m/e 301 (Et) and m/e 301 (Me)/m/e 280 were determined. A standard curve using authentic TXB_2 (0 to 20 ng) was constructed for each experiment. The assay will reliably detect 2.5 ng TXB_2 generated in 2.5 ml PRP. Basal levels of TXB_2 in the plasma ranged between < 0.5 and 2.5 ng ml^{-1}. The arachidonic acid challenge produced between 50 and 400 ng ml^{-1} TXB_2, depending on the particular donor used.

Preparation of prostaglandin H_2 and thromboxane A_2

For the chemical preparation of PGH_2 from $PGF_{2\alpha}$ and the enzymatic conversion of PGH_2 to TXA_2 by horse platelet microsomes methods described by Armstrong *et al.* (1983) were followed. It should be stressed that particulate matter in the thromboxane generating system was removed by rapid filtration at 0°C through an Amicon MPS-1 micropartition unit before addition to the platelet suspension.

Compounds

Stock solutions of prostanoids were prepared in ethanol (usually 5 or 10 mg ml^{-1}) and stored at −20°C. Aqueous solutions of the corresponding sodium salts were prepared either by evaporating the ethanolic solution with a nitrogen jet and dissolving the residue in warm, 0.9% NaCl/0.05% $NaHCO_3$ solution (water soluble compounds) or by adding the calculated molar equivalent of NaOH, evaporating the solution and dissolving the residue in warm saline (lipid soluble compounds).

EP 011 (*rac* 5-*endo*(6′-carboxyhex-2′Z-enyl)-6-*exo*(4″-p-flurophenoxy-3″ R-hydroxybut-1″ E-enyl)-bicyclo[2.2.1]hept-2-ene) and PTA_2(2-*exo*-6′carboxy-hex-2′Z-enyl)-6,6-dimethyl-3-*endo*-(3″S-hydroxyoct-1″E-enyl)-bicyclo[3.1.1.] heptane) were prepared as described by Wilson *et al.* (1982). A sample of PTA_2 (natural enantiomer) was also obtained from Dr M.P.L. Caton of May & Baker Research Laboratories, Dagenham, Essex. The biological profiles of the two products were identical. EP 045 (*rac*

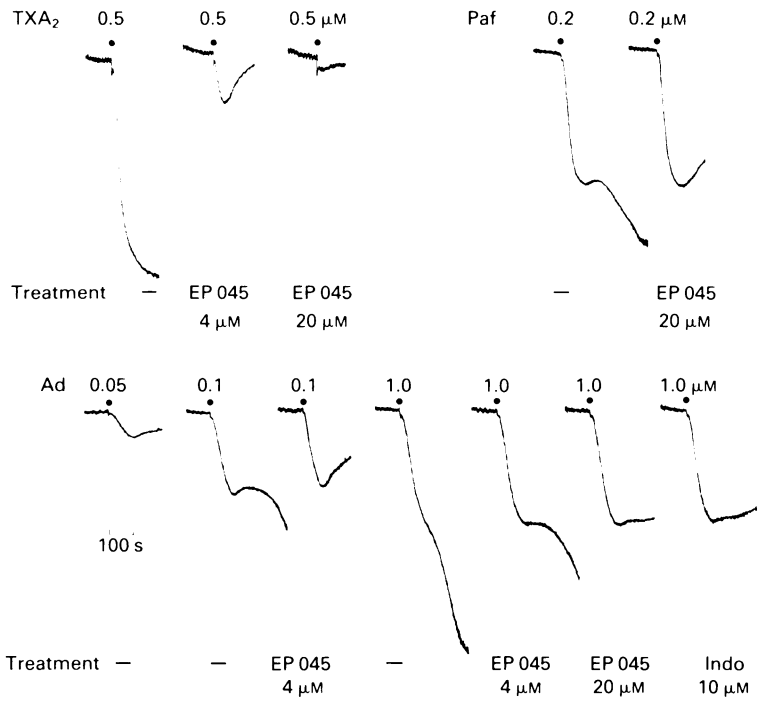

Figure 2 Effect of EP 045 on aggregation responses in diluted PRP induced by thromboxane A_2 (TXA_2) (incubation of prostaglandin H_2 (PGH_2) with horse platelet microsomes), platelet activating factor (Paf) and adrenaline (Ad). Final concentrations in the cuvette are shown. EP 092 produces similar effects at concentrations one-twentieth those of EP 045. Indo = indomethacin.

5-*endo*(6'-carboxyhex-2'Z-enyl)-6-*exo*[N-phenylcarbamoyl)-hydrazonomethyl]-bicyclo[2.2.1]heptane) and EP 092 (*rac* 5-*endo*(6'-carboxyhex-2'Z-enyl)-6-*exo*{1''-[N-(phenylthiocarbamoyl)hydrazono]ethyl}-bicyclo[2.2.1]heptane) were prepared from precursors described by Wilson *et al.* (1982). *rac* 2-*exo*(6'-Carboxyhex-2'Z-enyl)-3-*endo*(3'' S-hydroxyoct-1'' E-enyl)-7-oxabicyclo[2.2.1]-heptane, referred to in the text as the '9,11-endoxy analogue', was prepared in our laboratory: the Squibb research group (Sprague *et al.*, 1983) have attributed the platelet aggregating activity of this racemate to the enantiomer with the natural prostane skeleton, SQ 26655. The 16-*p*-fluorophenoxy-17, 18, 19, 20-tetranor analogue of $PGF_{2\alpha}$ was also prepared in our laboratory: ICI 79939 (Dukes *et al.*, 1974) is the racemic form of this compound and was used by us in all previous studies.

CTA_2 (natural enantiomer, Ansell *et al.*, 1982) was a gift from May & Baker Pharmaceuticals. 11,9-Epoxymethano-PGH_2, 9,11-azo-PGH_2, TXB_2, PGD_2, PGE_1 and $PGF_{2\alpha}$ (all natural enantiomers) were gifts from the Upjohn Company, U.S.A. ZK 36374 was a gift from Schering AG, Bergkamen-Berlin. 3,3,4,4-Tetradeutero-$PGF_{2\alpha}$ was purchased from the Stable Isotope Products section of Merck, Sharp & Dohme Canada Ltd.

Indomethacin was donated by Merck, Sharpe & Dohme Ltd, fluribiprofen by Boots Ltd, and dazoxiben by Pfizer Ltd. Platelet activating factor (Paf) was purchased from the Sigma Chemical Co. Ltd.

Results

EP 045 and EP 092

Specificity of the inhibitory effects

Although both compounds are potent inhibitors of thromboxane-induced aggregation in diluted PRP they do not inhibit either the rate or final magnitude of primary aggregation waves induced by ADP, Paf and adrenaline (Figure 2); the highest concentration tested was 20 μM for EP 045 and 5 μM for EP 092. In addition neither compound raised cyclic AMP levels. In three experiments on diluted PRP the cyclic AMP content in the presence of 12.5 μM EP 045 was 0.98, 0.98 and 0.99 × basal and with 25 μM EP 045 0.98, 0.99 and 1.01 × basal. Results for EP 092 are shown in Figure 4. It appears unlikely therefore that physiological antagonism though elevation of cyclic AMP levels contributes to the thromboxane block produced by EP 045 and EP 092.

Second phase aggregation responses to ADP, Paf and adrenaline are inhibited by EP 045 and EP 092 (Figure 2). The maximum effect achieved is always similar to that found with cyclo-oxygenase inhibitors such as flurbiprofen and indomethacin (both 10 μM). However, neither EP 045 (1–20 μM) nor EP 092 (1.25–6.25 μM) inhibited the conversion of arachidonic acid to TXA_2. At 12.5 μM EP 092 inhibited TXA_2 production by 21, 33 and 35% in platelets from three different donors. In two experiments flurbiprofen at 10 μM inhibited TXA_2 production by 97%. Inhibition by EP 045 and EP 092 of the endogenous thromboxane component of aggregation is therefore likely to be due to thromboxane receptor block rather than to inhibition of biosynthesis.

Discrimination between different thromboxane-like agonists

In six experiments using diluted PRP from different donors, PGH_2 at a concentration of 0.1 μM produced a shape change response, at 0.25 and 0.5 μM a reversible aggregation wave and at 1.0 μM and above a rapid irreversible aggregation wave. EP 045 at 4 μM produced a partial block of the irreversible wave induced by 1 μM PGH_2; at 20 μM complete inhibition was obtained (Table 1). In one of these experiments the thromboxane synthetase inhibitor dazoxiben (Randall *et al.*, 1981) at concentrations of 30 and 300 μM had no effect on the irreversible aggregation response to 1 μM PGH_2. In two further experiments PGH_2 (0.5 to 5 μM) gave concentration-dependent reversible aggregation

Table 1 Inhibition by EP 045 of platelet aggregation induced by prostaglandin H_2 (PGH_2), thromboxane A_2 (TXA_2) and 11, 9-epoxymethano-PGH_2

		% inhibition of aggregation			
Treatment		*PGH_2 1.0 μM irreversible*	*PGH_2 5.0 μM reversible*	*TXA_2 0.5 μM PGH_2/HPM irreversible*	*11,9-epoxymethano-PGH_2 0.5 μM irreversible*
EP 045	4 μM	42,73,74	not done	75,90	84,85,93
EP 045	20 μM	100 (n = 6)	100 (n = 2)	100 (n = 3)	100 (n = 6)

Diluted platelet-rich plasma (PRP) was used. Values for individual experiments are given, except when complete inhibition was always produced. The aggregation response was taken as the maximum pen deflection occurring within the first 100 s. HPM = horse platelet microsomes.

Table 2 Inhibition by EP 045 of platelet aggregation induced by stable thromboxane mimetics

Aggregating agent	*Dose ratios with EP 045 as antagonist* 4 μM	20 μM
11,9-epoxymethano-PGH_2	4.6 ± 0.4	19.6 ± 0.5
	(n = 6)	
9,11-azo-PGH_2	3.3 ± 0.4	13.8 ± 0.6
	(n = 4)	
9,11-endoxy analogue	4.4 ± 1.0	17.3 ± 1.0
	(n = 4)	
16-p-fluorophenoxy-17,18,19,20-tetranor-$PGF_{2\alpha}$	3.0 ± 0.3	9.9 ± 0.9
	(n = 4)	
16-p-fluorophenoxy-17,18,19,20-tetranor-9,11-etheno-PGH_2 (EP 011)	2.7 ± 0.3	9.6 ± 0.9
	(n = 4)	

Diluted platelet-rich plasma (PRP) was used. Values represent means ± s.e.mean.

waves: EP 045 at 20 μM abolished the response to the addition of 5 μM PGH_2.

Incubation of PGH_2 with a suspension of horse platelet microsomes at 0°C afforded a solution of TXA_2 for immediate use in the aggregation experiments. TXA_2 derived from 0.5 μM PGH_2 produced irreversible aggregation in diluted PRP. EP 045 at 4 μM produced a marked inhibition of this response and at 20 μM complete inhibition (Table 1 and Figure 2). For comparison, the inhibitory effect of EP 045 on the irreversible aggregation produced by 0.5 μM 11,9-epoxymethano-PGH_2 (the standard agonist in our studies) is included in Table 1. It is apparent that EP 045 shows similar blocking activity against both the natural and synthetic agonists.

In further experiments the blocking activity of EP 045 against 11,9-epoxymethano-PGH_2 and four other synthetic prostanoids of quite different chemical structure was measured (Table 2). Each analogue is a full agonist in the sense that it can produce a rapid

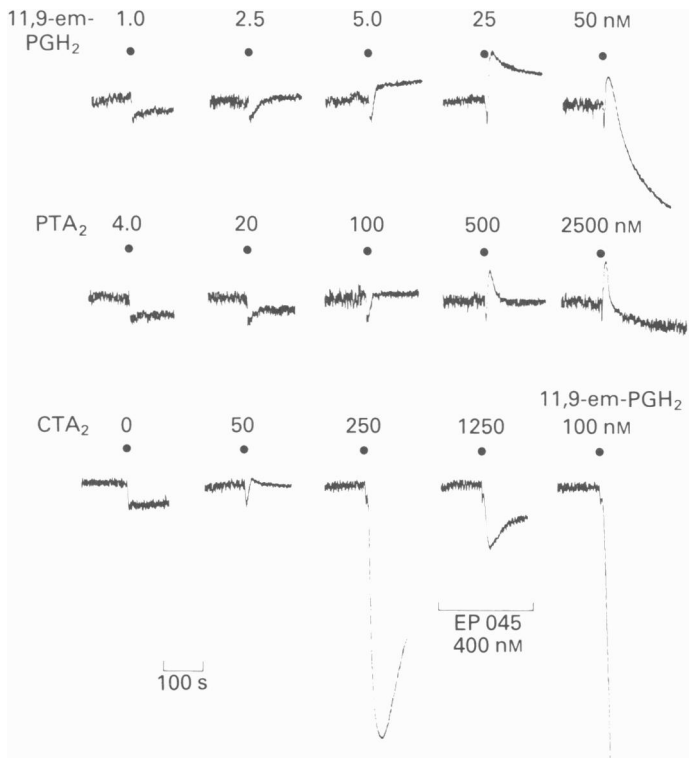

Figure 3 Shape change/aggregation responses to 11,9-epoxymethano-prostaglandin H_2 (11,9-em-PGH_2) PTA_2 and CTA_2 in a plasma-free suspension of human platelets. Final concentrations are shown. The sharp downward deflection of the pen recording on addition of the agonist is due to dilution of the suspension. The subsequent upward deflection (decrease in light transmission) with some loss of the random oscillation is caused by the platelet shape change. CTA_2 at concentrations between 0.5 and 10 μM produces a rapidly reversing aggregation wave similar in magnitude to that seen with 250 nM above. The full magnitude of the irreversible wave produced by 100 nM 11,9-epoxymethano-PGH_2 is about 3 times the deflection shown above.

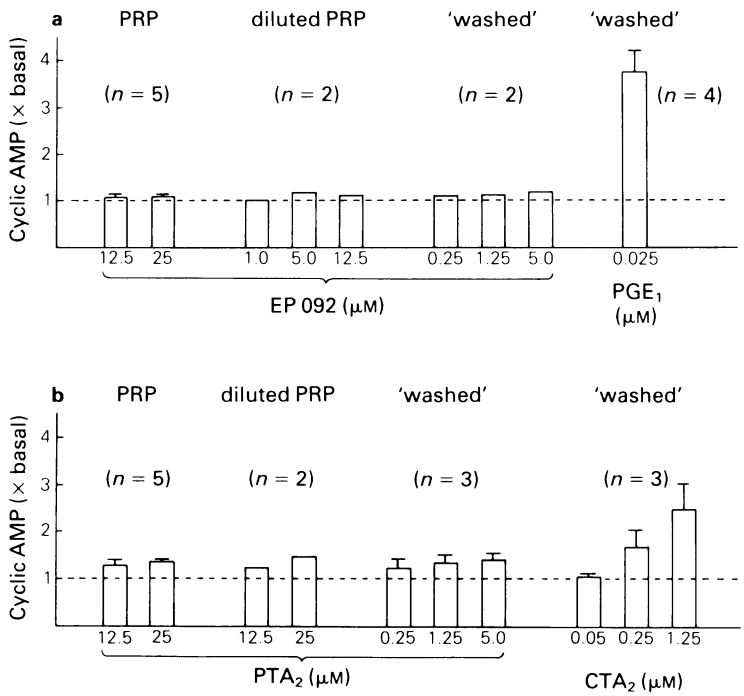

Figure 4 Effect of (a) EP 092 and prostaglandin E_1 (PGE_1) (b) PTA_2 and CTA_2 on cyclic AMP levels in human platelet experiments. Each column represents the mean and vertical lines show s.e.means of *n*, number of donors used.

irreversible aggregation wave similar to that shown for TXA_2 in Figure 2. EP 045 produced parallel rightward shifts of the log concentration-response curve for each agonist and irreversible aggregation could always be achieved by raising the agonist concentration. Calculation of dose-ratios shows that the two 16-*p*-fluorophenoxy analogues are slightly more resistant to the blocking action of EP 045 than the three analogues with a natural ω-chain.

The final stable analogue studied, CTA_2, bears the closest structural similarity to TXA_2 (Figure 1). However, it produces only reversible aggregation in human PRP (Armstrong *et al.*, 1983; Burke *et al.*, 1983). If CTA_2 is added simultaneously with 11,9-epoxymethano-PGH_2 the aggregation response is less than additive, whereas addition with ADP results in irreversible aggregation. We had interpreted these effects to be due to a partial agonist action of CTA_2 at the thromboxane receptor. However, we have subsequently found that CTA_2 raises cyclic AMP levels in human platelets. Unfortunately our stocks of CTA_2 were small by this time. We therefore made repeated measurements on washed platelets where CTA_2 has a similar profile of activity but is more active due to the absence of plasma protein binding. Figure 3 shows the rapid reversal of the primary aggregation wave typical of CTA_2 ($n = 4$). In Figure 4 the stimulant action of CTA_2 on cyclic AMP production is shown. EP 045 blocks both the shape change and reversible wave produced by CTA_2. With 0.4 μM EP 045 present, a 10 fold increase in the CTA_2 concentration is required to produce a response matching that of the control. This degree of block is slightly greater than that seen when 11,9-epoxymethano PGH_2 is used as the agonist (see later). With 4 μM EP 045 complete block of shape change and aggregation induced by CTA_2 is produced.

Inhibition of the release reaction directly mediated by 11,9-epoxymethano-PGH_2 Both irreversible aggregation and 5-HT release induced by 11,9-epoxymethano-PGH_2 in diluted PRP are predominantly direct actions, as shown by the relatively small inhibitory effect (15–20% reductions in 3 donors) of the cyclo-oxygenase inhibitor flurbiprofen (10 μM). In the same experiments EP 045 inhibited aggregation and release induced by 11,9-epoxymethano PGH_2 in a parallel manner and the block could be surmounted by raising the agonist concentrations (Figure 5).

It is of interest to compare the inhibitory effect of the chemically stable PGI_2 analogue ZK 36374 (Skuballa & Vorbrüggen, 1983 and references therein). Its potent agonist action on the PGI_2 receptor results in elevation of cyclic AMP and a consequent physiological antagonism. This is typified by an

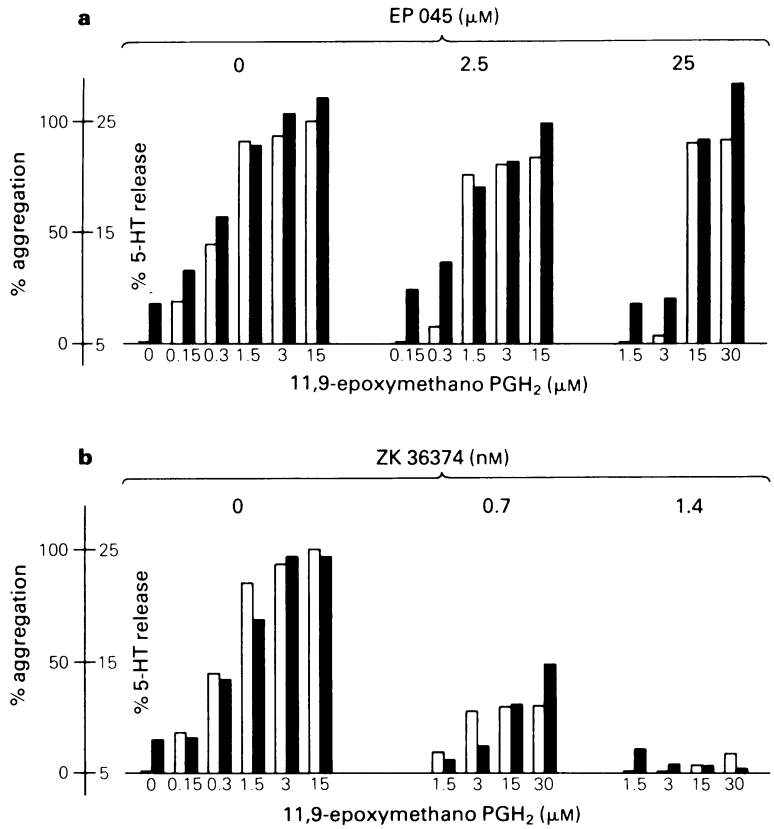

Figure 5 11,9-Epoxymethano-prostaglandin H_2 (PGH_2) concentration-response relationships for aggregation (open columns) and 5-hydroxytryptamine (5-HT) release (solid columns) in diluted PRP. The inhibitory effects of (a) EP 045 and (b) ZK 36374 are shown.

insurmountable block of both aggregation and release induced 11,9-epoxymethano-PGH_2 (Figure 5).

Affinity constant determination

Using washed platelets an attempt was made to determine the affinity constants of EP 045 and EP 092 for the thromboxane receptor. 11,9-Epoxymethano-PGH_2 was used as the agonist. With EP 045 present, the profiles of aggregation waves produced by the agonist were similar to control responses and the log concentration-response curves were shifted to the right in a parallel manner. Dose-ratios corresponding to concentrations of 0.2, 0.8 and 4 μM were 2.80 ± 0.33 (mean ± s.e.mean, $n = 4$), 11.0 ± 0.64 ($n = 5$) and 50.1 ± 7.6 ($n = 5$). Substitution of these data in the Schild equation (log (dose-ratio − 1) = [A]K_A) followed by least squares regression analysis gave a correlation coefficient (r) of 0.973 and a slope of 1.09 ± 0.16 (95% confidence limits). The latter value is not significantly different from unity (Student's $t = 1.26$, theoretical $t = 2.18$ at $P = 0.05$) and this suggests that the antagonism is competitive in nature. When the regression line is constrained to a slope of 1.00, an affinity constant of $1.1 \times 10^7\ M^{-1}$ is obtained for EP 045.

With 0.02 μM EP 092 present the character of the aggregation waves was identical to that of the controls and the agonist log concentration-response curves were parallel. A dose-ratio of 2.50 ± 0.25 ($n = 3$) was obtained. With 0.05 μM EP 092 the rate of primary wave aggregation was marginally slower but reversible aggregation still peaked within 100 s of agonist addition. Again the log concentration-response curves were parallel giving a dose-ratio of 7.13 ± 1.64 ($n = 4$). At the two highest concentrations, 0.10 and 0.25 μM, greater block was produced but the character of the aggregation waves differed considerably from that of the control. The onset of the primary aggregation wave was slowed but there was a greater tendency to proceed to irreversible aggregation, such that the distinction between the primary and secondary waves

Table 3 Effect of EP 045 and EP 092 on the anti-aggregatory activity of prostaglandin D_2 (PGD_2), PGE_1 and ZK 36374

Inhibitor	IC_{50} values for inhibition of ADP-induced aggregation (nM) Control	EP 045 (20 μM)	Mean dose-ratio	IC_{50} values for inhibition of ADP-induced aggregation (nM) Control	EP 092 (20 μM)	Mean dose-ratio
	16.8	20.1		17.0	17.0	
PGD_2	24.0	27.8	1.15	24.1	28.4	1.08
	42.6	47.7		49.0	52.0	
	9.2	9.3		22.7	24.7	
PGE_1	13.8	13.6	0.97	35.5	39.8	1.10
	58.8	53.7		36.9	40.0	
	0.58	0.43		0.77	0.78	
ZK 36374	0.53	0.42	0.85	0.94	0.99	1.04
	0.45	0.48		1.13	1.18	

The concentrattion of ADP was 1 μM or 2 μM. Indomethacin (10 μM) was present in all experiments.

became less clear. It was not possible to devise a satisfactory method of quantifying the two types of aggregation profiles so that dose-ratios, meaningful in terms of the Schild analysis, could be obtained. It was apparent, however, that the degree of block with the higher two concentrations of EP 092 was somewhat greater than would be predicted by application of the Schild equation to the data obtained with the two lower concentrations.

Effect on the inhibitory activities of PGD_2, PGE_1 and ZK 36374

In the presence of 10 μM indomethacin, ADP at 1 μM or 2 μM was used to produce irreversible aggregation in diluted PRP. Log concentration-inhibition curves were constructed for PGD_2, PGE_1 and ZK 36374 in the presence and absence of either EP 045 or EP 092 (both 20 μM). The concentrations required for 50% inhibition of the ADP response were calculated and are shown in Table 3. It is clear that EP 045 and EP 092 do not inhibit the anti-aggregatory effects of the three prostaglandin analogues.

Pinane thromboxane A_2

In diluted PRP from several donors the addition of PTA_2 (5–20 μM) gave a small, transient increase in light scattering. We were unsure as to whether this response reflected a change in platelet shape and proceeded to examine the compound for thromboxane antagonism. At 2 μM PTA_2 produced a parallel rightward shift of the log concentration-response curve for 11,9-epoxymethano-PGH_2 (dose-ratio = 2.35 ± 0.23, $n = 4$ donors) but did not affect primary aggregation waves induced by ADP (1 μM) or Paf (0.1 μM). At 10 μM PTA_2 a marked inhibition of the thromboxane mimetic was observed and the log concentration-response curve was flattened (dose-ratio > 10, $n = 4$). However, there was also a small inhibition of ADP-induced aggregation (4, 5 and 7%) and a greater effect against Paf (10, 33 and 47% inhibition respectively). At 25 μM PTA_2 inhibited Paf-induced aggregation by 19, 65 and 75% respectively. These relatively weak inhibitory effects of PTA_2 against the non-thromboxane aggregating agents could be correlated with small increases in cyclic AMP levels (Figure 4).

When PTA_2 was subsequently examined on the washed platelet suspension a shape change response was consistently observed (Figure 3). In seven experiments a threshold effect was obtained with 20 nM and a half-maximal response with 137 ± 31 nM. The PTA_2 log concentration-response curve was always slightly shallower than that for 11, 9-epoxymethano-PGH_2 (threshold effect at 1 nM, half maximal shape change at 4.0 ± 0.6 nM). In four of the experiments PTA_2 at concentrations between 500 and 2500 nM gave large shape change responses which rapidly faded, and the random oscillations typical of the resting discoid state were regained (Figure 3). In two other experiments it appeared that the shape change had been overtaken by a small aggregation wave. Small increases in cyclic AMP were found when washed platelets were exposed to these high concentrations of PTA_2 (Figure 4). EP 045 at 1 μM completely blocked shape change responses to PTA_2 (100–2500 nM) in three experiments.

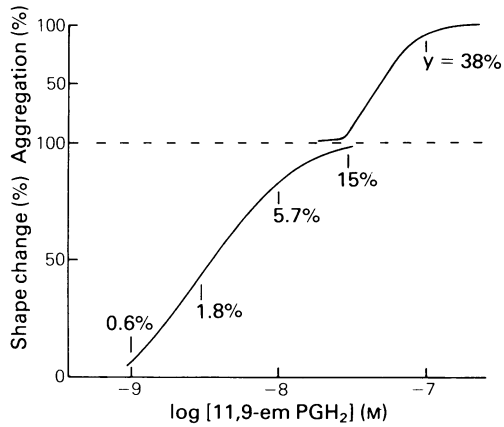

Figure 6 Typical log concentration-response relationships for both shape change and aggregation induced by 11, 9-epoxymethano-prostaglandin H_2 (11,9-em-PGH_2) in washed human platelets. From ligand binding studies (Armstrong *et al.*, 1983) the predicted occupancies of the agonist at concentrations of 1, 3, 10, 30 and 100 nM are shown. There are about 1,700 specific thromboxane binding sites per platelet and a threshold shape change corresponds to occupation of 10 of these sites.

Discussion

The evidence obtained with EP 045 and EP 092 tends to indicate that a single receptor type mediates the thromboxane-sensitive shape change, primary aggregation and release reaction of human platelets. EP 045 and EP 092 specifically block this thromboxane receptor, having little affinity for receptors mediating the pro-aggregatory actions of ADP, Paf and adrenaline and the anti-aggregatory actions of PGI_2 and PGD_2. In the case of EP 045 the Schild analysis supports a competitive type of antagonism. With the more active EP 092 the situation is less clear cut. One explanation of the delaying effect of high concentrations of EP 092 on the primary aggregation waves to 11,9-epoxymethano-PGH_2 which is consistent with simple competition is that the rate of dissociation of the antagonist from the thromboxane receptor is slow in relation to the time course of aggregation. Thus the agonist, immediately after addition, can only attach to unoccupied receptors and some dissociation of EP 092 is required before the complete response can be produced. In practical terms, this effect will only be of consequence if the agonist must occupy a considerable proportion of the total population of thromboxane receptors to achieve aggregation (see Stephenson, 1956). Our ligand binding studies with washed human platelets (Armstrong *et al.*, 1983) are a source of information regarding the receptor occupancy of 11,9-epoxymethano-PGH_2 at concentrations which produce shape change and aggregation. From the displacement curve for 11,9-epoxymethano-PGH_2 ($[^3H]$-9,11-epoxymethano-PG-H_2 was the radio-ligand) an affinity constant (K_A) of approximately $6 \times 10^6\ M^{-1}$ can be obtained. In Figure 6 the calculated occupancies ($y = [A]K_A/1 + [A]K_A$) at appropriate concentrations of 11,9-epoxymethano-PGH_2 are shown. It is clear that, although shape change can be elicited with low receptor occupancies, at least 15% occupancy is required for threshold aggregation and about 40% for irreversible aggregation. The standard agonist therefore has little receptor reserve in this situation. It is of relevance that we only see a delayed aggregation response with the more potent of the thromboxane receptor antagonists available to us: a small dissociation rate constant is presumably a major factor contributing to the high affinity of each antagonist. Ligand binding measurements with radiolabelled EP 092 may provide information on the kinetics of the interaction between EP 092 and the thromboxane receptor.

In agreement with previous work (Nicolaou *et al.*, 1979) we have found that PTA_2 effectively blocks aggregation produced by thromboxane mimetics but has little activity against ADP-induced aggregation. However, this evidence alone is not sufficient to exclude a contribution to the block through functional antagonism. We have shown (Armstrong *et al.*, 1984) that the ability of PGI_2, PGD_2 and their mimetics to inhibit platelet aggregation depends on the aggregating agent used. It is easier to inhibit the action of a thromboxane-like agonist than either Paf or thrombin, and ADP is the most difficult agent to inhibit. The ability of high concentrations of PTA_2 to inhibit primary aggregation responses to Paf therefore indicates that functional antagonism can augment its thromboxane blocking action. The weak stimulant action of PTA_2 on cyclic AMP production supports this conclusion.

In the plasma-free platelet suspension we have shown that PTA_2 is not a pure antagonist. We suggest that PTA_2 is probably a partial agonist at the platelet thromboxane receptor. However, the rapid reversal of the shape change response obtained with high concentrations of PTA_2 may reflect its ability to raise cyclic AMP levels.

We are now in a position to ask how the thromboxane receptor of the human platelet compares with corresponding receptors in smooth muscle. Some relevant information on the rabbit aorta, dog saphenous vein, and guinea-pig trachea (Jones *et al.*, 1982) together with data on washed human platelets from this study is presented in Table 4.

Natural agonists

The instability of PGH_2 and TXA_2 limits their usefulness in this type of exercise. This is particularly true of

Table 4 A comparison of some thromboxane-sensitive systems

	Preparation			
	Rabbit aorta	*Dog saphenous vein*	*Guinea-pig trachea*	*Human platelets**
Natural agonists				
TXA_2	potent full agonist, blocked by EP 045 and EP 092			
PGH_2	conversion to other natural prostaglandins makes comparisons difficult thromboxane-like agonist effects are blocked by EP 045 and EP 092			
Synthetic mimetics				
11,9-epoxymethano-PGH_2	EPMR = 1.0 (threshold = 1.5 nM)	1.0 (1.0 nM)	1.0 (3.5 nM)	1.0 (1.0 nM)
9,11-azo-PGH_2	1.4	1.2	1.1	0.20
9,11-endoxy analogue	0.65	0.59	0.41	0.49
ICI 79939	3.1	4.0	0.90†	3.6¶
EP 011	0.12	0.087	0.055	0.17
CTA_2	0.99 (threshold = 1.5 nM)	partial agonist? (4 nM)	partial agonist? (6 nM)	partial agonist? (10 nM)
PTA_2	partial agonist (max = 47–60%; (threshold = 30 nM)	partial agonist (24–36%; 10 nM)	partial agonist (4–13%; –)	partial agonist (shape change only; 20 nM)
Competitive antagonists				
EP 045	K_B‡ $= 2.0 \times 10^6$	1.5×10^7	3.2×10^7	1.1×10^7
EP 092	$= 1.8 \times 10^7$	8.7×10^7	9.2×10^7	$\sim 9 \times 10^7$

Equipotent molar ratios (EPMR) are means of at least three determinations. Threshold concentrations relate to a contraction equal to 5% of the maximum response to 11,9-epoxymethano PGH_2 on the smooth muscle preparations, and to the minimum detectable shape change in the aggregation system. On the guinea-pig trachea it is difficult to estimate a threshold concentration for PTA_2 due to the very shallow log concentration-response curve and the slow onset of the agonist action.

* washed platelets

† PGE-like contractile activity of ICI 79939 contributes to the measured potency (Jones *et al.*, 1982).

¶ Activity of the natural enantiomer.

‡ K_B = affinity constant (M^{-1}) using 11,9-epoxymethano PGH_2 as agonist obtained from a Schild plot.

PGH_2 since it can be converted to other prostaglandins (e.g. PGD_2 and PGE_2) which may have actions opposing those of PGH_2 itself. Nevertheless, TXA_2 is a potent full agonist in each of the four situations and its action and the thromboxane-like actions of PGH_2 are completely blocked by both EP 045 and EP 092.

Synthetic thromboxane mimetics

The compounds investigated show considerable diversity of structure. If one were to use equipotent molar ratios obtained on diluted PRP then significant differences between the rank order of potency on the human platelet and those on the smooth muscle preparations are seen. However, if equipotent molar ratios (EPMR) obtained on a plasma-free suspension of human platelets are used then the differences are much less. The activity of the highly lipophilic analogue EP 011 is increased on the washed platelets and it is the most active agonist in the four systems and indeed in all thromboxane-sensitive systems that we have examined.

The thromboxane-like activity of CTA_2 requires careful consideration. It has been claimed that CTA_2 can be used 'to dissociate vasoconstrictor and platelet aggregatory actions of thromboxane' since 'it is the most potent coronary constrictor of the known prostanoids and yet, possesses no intrinsic ability to aggregate platelets' and 'is an inhibitor of arachidonic acid- and endoperoxide-induced platelet aggregation' (Lefer *et al.*, 1980). Our results are at variance with these statements. CTA_2 caused shape change and reversible aggregation in both the washed platelet suspension and diluted PRP and these effects were blocked by EP 045. In addition, we have shown that CTA_2 raises cyclic AMP levels: this effect undoubtedly contributes to the rapid reversal of aggregation seen with CTA_2. If it were possible to annul the cyclic AMP stimulant activity of CTA_2 then the analogue may indeed cause irreversible aggregation. We are current-

ly investigating this strategy. On rabbit aorta CTA_2 is a potent full agonist (Table 4). On dog saphenous vein and guinea-pig trachea it produces contractile responses of at least $\frac{2}{3}$ the 11,9-epoxymethano-PGH_2 maximum, but above this the log concentration-response curves become shallow: at this time we cannot ascertain whether CTA_2 is a partial agonist at the thromboxane receptor in these two preparations or whether a distinct inhibitory action of CTA_2 at the higher concentrations opposes a full thromboxane-like agonist action. Nevertheless, it can be seen from Table 4 that the threshold concentrations for the thromboxane mimetic activity of CTA_2 are in the 1–10 nM range on all four preparations.

The partial agonist action of PTA_2 is a feature common to the four preparations (and others such as the bullock iris sphincter, Dong & Jones, 1982). We suggest that the classification of PTA_2 as a thromboxane receptor antagonist does not accurately describe its interaction with thromboxane-sensitive systems.

Competitive thromboxane antagonists

We have commented previously on the lower affinity of EP 045 for thromboxane receptors in rabbit aorta relative to values for the dog saphenous vein and guinea-pig trachea (Jones *et al.*, 1982). A similar difference is seen with the higher affinity antagonist EP 092 and a large number of our EP analogues with thromboxane blocking activity. The affinity constant for EP 092 on human platelets ($9 \times 10^7 M^{-1}$) was calculated from experiments using 0.02 and 0.05 μM of the antagonist where the agonist log concentration-response curves were parallel and dose-ratios were less than 10. It is clear that the affinities of EP 045 and EP 092 for thromboxane receptors on washed human platelets are similar to those found for dog saphenous vein and guinea-pig trachea.

In conclusion, we suggest that the thromboxane receptor mediating aggregation in human platelets has considerable similarity with the corresponding receptors mediating contractions in vascular and respiratory smooth muscle.

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