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Ligand binding to thromboxane receptors on human platelets: correlation with biological activity

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1 The preparation of enantiomerically pure [^{3}H]-15 (**S**) 9, 11-epoxymethano PGH_2 (a thromboxane A_2-like agonist) has enabled the binding of ligands to the thromboxane receptor of the human platelet to be studied.

2 The binding of the radio-ligand to washed human platelets has 3 components. One component is not displaceable by 'cold' 9, 11-epoxymethano PGH_2 and its concentration-binding plot is roughly linear. The other 2 components are displaceable and saturable, and the larger of the two, which is sensitive to the stereochemistry of the C15 secondary alcohol, appears to represent the thromboxane receptor. About 1700 15(**S**)9, 11-epoxymethano PGH_2 molecules are specifically bound to a single platelet and 50% of this binding is achieved with a concentration of 75 nM.

3 Displacement of [^{3}H]-15(**S**)9, 11-epoxymethano PGH_2 is effected by (a) TXA_2 and PGH_2 and a number of bicyclic stable analogues (e.g. 9,11-azo PGH_2), all of which produce irreversible aggregation of human platelets; (b) analogues of $PGF_{2\alpha}$ with potent thromboxane-like activity (e.g. ICI 79939); (c) compounds with partial agonist activity on the platelet thromboxane system (e.g. CTA_2); (d) Thromboxane/endoperoxide analogues which specifically antagonize thromboxane-like actions on the human platelet (e.g. PTA_2 and EP 045).

4 Displacement is not achieved with the natural prostaglandins PGE_2, PGD_2 and $PGF_{2\alpha}$. Neither the thromboxane-synthetase inhibitor dazoxiben nor **R**(+)-trimethoquinol have high displacing activity.

5 The correlation of radio-ligand displacement with the biological activity of the competing ligands is discussed in relation to the nature of the thromboxane receptor on the human platelet.

Introduction

Thromboxane A_2 (TXA_2) is a potent inducer of aggregation and the release reaction in blood platelets (Hamberg, Svensson & Samuelsson, 1975; Svensson, Hamberg & Samuelsson, 1975) and is the predominant metabolite of arachidonic acid (AA)-derived prostaglandin endoperoxides PGG_2/H_2 in human platelets (Hamberg & Samuelsson, 1974; Hamberg, Svensson & Samuelsson, 1974). TXA_2 has a strained acetal structure and is very acid labile; under physiological conditions it has a half life of about 30 s. This instability has been the stimulus for the chemical synthesis of more stable compounds with potential thromboxane-like activity. Some of the compounds have ring structures close to that of TXA_2 and involve for example the replacement of both acetal oxygen atoms with sulphur (Hamanaka, Ohuchida & Hayashi, 1983) or one with sulphur and the other with a methylene unit (Ohuchida, Hamanaka & Hayashi, 1981).

Other highly active aggregating agents with thromboxane A_2-like actions, for example 9,11- and 11,9-epoxymethano (Bundy, 1975) and 9,11-azo (Corey, Narasaka & Shibazaki, 1976) analogues of PGH_2, are structurally more akin to the prostaglandin endoperoxides than to TXA_2 itself (Figure 1). It has also been shown that analogues with PGD, PGE or PGF ring systems can induce aggregation of human platelets particularly if they contain a gem-dimethyl group at C 16 or a 16-*p*-halophenoxy-17, 18,19,20-tetranor structure (Jones & Marr, 1977; MacIntyre & Gordon, 1977; Jones, Wilson & Marr, 1979). In order to know whether these compounds of quite diverse structure interact with a common receptor on the human platelet to produce their pro-aggregatory effects, we have synthesized a radio-labelled thromboxane mimetic, [^{3}H]-15(**S**)9,11-epoxymethano PGH_2, and studied the nature of its binding to washed human platelets and its displacement by thromboxane-like agonists.

Several groups have recently reported the synth-

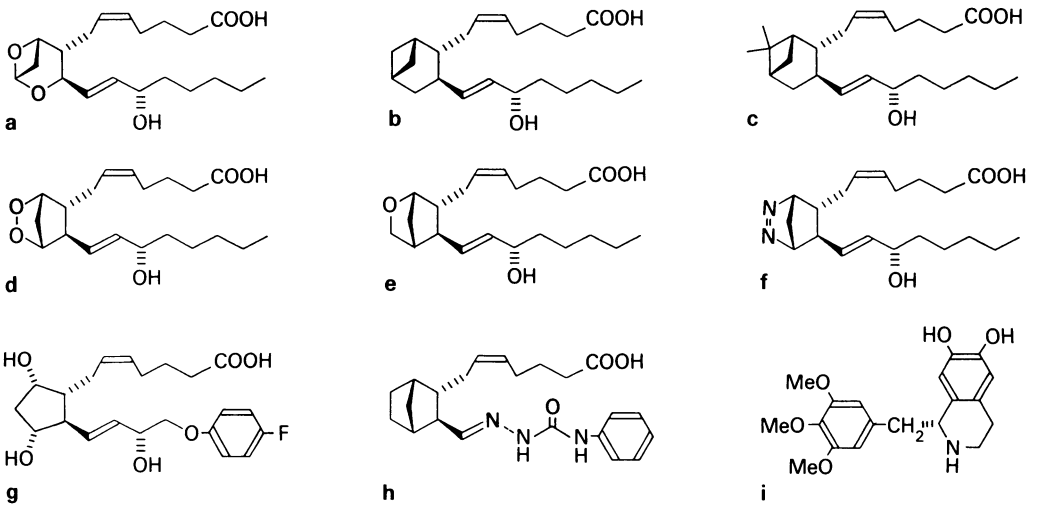

Figure 1 Structures of some of the compounds used in this study: (a) TXA_2, (b) CTA_2, (c) PTA_2, (d) PGH_2, (e) 9,11-epoxymethano PGH_2, (f) 9,11-azo PGH_2, (g) ICI 79939, (h) EP 045, (i) **R**(+)-trimethoquinol. The radio-ligand is the 15[^{3}H] derivative of compound (e). Compounds (a) to (f) are pure enantiomers, each having the natural prostane structure. Compounds (g) and (h) are racemates and the enantiomer with the natural prostane structure is shown. Using the Cahn-Ingold-Prelog convention, compounds (a) to (f) have the **S** configuration at C15 (groups in descending order of precedence are OH, CH = CH-, CH_2-CH_2-). The 15-hydroxyl group in compound (g) as drawn above has the same orientation relative to the carbon chain as the 15-hydroxyl groups in compounds (a) to (f). However, because the order of precedence is OH, CH_2-O-, CH = CH- the configuration at C15 is designated **R** (see March, 1977).

esis of thromboxane or prostaglandin analogues which inhibit the aggregatory action of TXA_2 and its stable mimetics without affecting the primary wave induced by adenosine diphosphate (ADP). These agents, which by pharmacological criteria appear to act as thromboxane receptor antagonists, include pinane thromboxane A_2 (PTA_2) (Nicolaou, Magolda, Smith, Aharony, Smith & Lefer, 1979), 13-azaprostanoic acid (Le Breton, Venton, Enke & Halushka, 1979) and EP 045 (Jones & Wilson, 1981; Jones, Wilson, Armstrong, Peesapati & Smith, 1983). We hoped that our ligand binding technique might indicate whether these compounds, and in particular EP 045, compete with thromboxane-like agonists for the same binding site (receptor) on human platelets.

Methods

*Preparation of [^{3}H]-15(**R**)-and [^{3}H]-15(**S**)9,11-epoxymethano PGH_2*

15(**S**)9,11-epoxymethano PGH_2 was prepared from natural PGA_2 as described by Bundy (1975). Oxidation with chromium trioxide in acetone (Jones Reagent) yielded the 15-ketone derivative which was supplied to Amersham Radiochemical Centre for reduction with tritiated sodium borohydride. The cruder [^{3}H]-9,11-epoxymethano PGH_2 was separated into 15(**S**) and 15(**R**) forms by liquid-gel partition chromatography: a 20 g N1114-20%-LH20 gel column was used and eluted with hexane/1,2-dichloroethane/ethanol (100/100/5 by vol.) containing 0.1% acetic acid. Purity was assessed by radio t.l.c. analysis. The specific activity of [^{3}H]-15(**S**)9,11-epoxymethano PGH_2 was estimated by a combination of bioassay on the dog saphenous vein against 'cold' 15(**S**)9,11-epoxymethano PGH_2 and conventional scintillation counting. The value of 13.9 Ci/mmol agreed with that determined by mass spectrometric analysis of the tritium/protium content, assuming that replacement of one hydrogen atom allows a maximum specific activity of 28.9 Ci/m atom. The [^{3}H]-15(**R**) epimer was assumed to have the same specific activity as the [^{3}H]-15(**S**) epimer.

Binding studies

All procedures were carried out at room temperature unless otherwise stated. Blood was withdrawn from the antecubital vein of healthy volunteers into CPD anticoagulant (14 ml 100 ml^{-1} blood) and centrifuged at 160 *g* for 20 min. CPD contains citric acid 0.33 g, sodium citrate 2.63 g, monosodium phosphate 0.22 g and dextrose 2.55 g per 100 ml. Platelet-

rich-plasma (PRP) was collected and treated with indomethacin (final concentration of 10^{-5} M) to inhibit the biosynthesis of prostaglandin endoperoxides and TXA_2. PGE_1 (1.7×10^{-8} M) was also added to aid resuspension of the platelet pellet. The PRP was centrifuged at 1600 *g* for 10 min and the resulting pellet suspended in a citrate medium (sodium chloride 81.82 g, potassium chloride 3.73 g, glucose 9.01 g, citric acid 12.08 g and sodium citrate 5.88 g/10 l, adjusted to pH 7.5 with 2 M Tris solution) to give a final platelet count in the region of 5×10^8 platelets ml^{-1}.

Unless otherwise stated, the [^{3}H]-15(**S**)9,11-epoxymethano PGH_2 ligand was used in the binding studies. A stock solution of 500 ng ml^{-1}, 20 μCi ml^{-1} of the tritiated ligand was prepared in methanol and appropriate amounts dispensed into Eppendorf tubes and the solvent evaporated. Platelet suspension (1 ml) was added to each tube, 6 tubes at a time, the contents mixed and then incubated at room temperature for a given period of time (usually 4 min). Incubation was terminated by centrifugation in an Eppendorf centrifuge at 15000 *g* for 2 min. The supernatant was rapidly removed with a Pasteur pipette. Any remaining supernatant was cleaned from the pellet with a 'cotton bud'. The pellet was digested by incubation with 1 M hyamine hydroxide (Fisons) at 50 °C for 5 min. A few drops of 2 M hydrochloric acid were added to each tube and the contents transferred to scintillation fluid (10.5 g PPO dissolved in 1.5 l toluene and 900 ml 2-ethoxyethanol). The acid serves to neutralize the strong alkali and prevents chemiluminescence interfering with the liquid scintillation counting. Samples were counted for 10 min.

In displacement studies, prostaglandins and analogues were added in saline simultaneously with the platelet suspension at the start of the incubation. When arachidonic acid was used as the competing agent, the platelets were not treated with indomethacin, but the PGE_1 concentration was raised to 3×10^{-7} M and the incubation period was shortened to 3 min.

Displacement by synthetic PGH_2 and by TXA_2 generated enzymatically from synthetic PGH_2 was also studied. $PGF_{2\alpha}$ was converted into 9β,11β-dibromo-9,11-dideoxy $PGF_{2\alpha}$ by the method of Porter, Byers, Holden & Menzel (1979). Treatment of the dibromo derivative with 90% H_2O_2/silver trifluoroacetate gave PGH_2 which was purified by silicic acid chromatography (10 g Unisil, 100–200 mesh Clarkson Chemical Co., USA; gradient elution, 20% ethyl acetate in hexane to pure ethyl acetate over 3 h; −20 °C). An estimate of mass was made by reduction of about 1 μg of PGH_2 to $PGF_{2\alpha}$ with $SnCl_2$ in aqueous ethanol, followed by GC/MS analysis using 3,3,4,4-tetradeutero $PGF_{2\alpha}$ (1.0 μg) as internal standard. The incubation period was again shortened to 3 min, and was carried out on ice.

For the generation of TXA_2 a given amount of PGH_2 in Krebs solution was mixed with a suspension of horse platelet microsomes (HPM), a rich source of the enzyme thromboxane synthetase (Moncada, Needleman, Bunting & Vane, 1976), and incubated for 30 s at 0 °C. The suspension was filtered rapidly at 0 °C under pressure from a nitrogen gas cylinder through an Amicon MPS-1 micropartition unit (Amicon Ltd, Stonehouse). Aliquots of the filtrate were immediately incubated with 1 ml of washed platelet suspension for 3 min at 0 °C. In parallel experiments using the superfused rabbit aorta the ability of the horse platelet microsomes to generate TXA_2 from PGH_2 was confirmed (Needleman, Moncada, Bunting, Vane, Hamberg & Samuelsson, 1976). A portion of the TXA_2 filtrate was added to 0.02 M HCl (to produce TXB_2) and extracted with diethyl ether. GC/MS analysis, using authentic TXB_2 as an external standard, showed that during the 30 s incubation period about 60% of the PGH_2 had been converted to TXA_2.

Platelet aggregation

Platelet aggregation was monitored optically using a modified Cary 118C spectrophotometer. Citrated human platelet-rich plasma (1 ml) was added to 1 ml Krebs solution and 0.4 ml 0.9% w/v NaCl solution (saline) in a 3 ml plastic cuvette. After incubation for 2 min at 37 °C with constant stirring (1000 rev min^{-1}), the aggregating agent was added in 0.1 ml saline. Inhibitory drugs were added 2 min before the addition of the aggregating agent (replacing all or part of the 0.4 ml saline).

Compounds

rac 15(**S**)9,11-Ethano PGH_2, *rac* 15(**S**)10a-homo-9,11-ethano PGH_2, *rac* 15(**R**)16-*p*-fluorophenoxy-17,18,19,20-tetranor-9,11-etheno PGH_2 (EP 011) and the 15(**R**) and 15(**S**) epimers of PTA_2 were prepared in our laboratory (Wilson, Peesapati, Jones & Hamilton, 1982). *rac* 5-*endo* (6'-Carboxyhex-2'Z-enyl)-6-*exo* (N-phenyl-carbamoylhydrazonomethyl)-*bicyclo* [2,2,1]-heptane (EP 045) and *rac* 5-*endo*(6'-carboxyhex-2'Z-enyl)-6-*exo*(carbamoylhydrazonomethyl) - *bicyclo* [2,2,1] - heptane (EP 033) were prepared from precursors described in Wilson *et al.*, (1982). 10α,11α-Oxido PGA_2 was prepared according to the method of Corey & Ensley (1973).

$PGF_{2\alpha}$, 15(**S**)15-methyl $PGF_{2\alpha}$, 15(**R**)16,16-dimethyl $PGF_{2\alpha}$, PGD_2, PGE_1, PGE_2, 11,9-epoxymethano PGH_2 and 9,11-azo PGH_2 were gifts from the Upjohn Co., USA. *rac* 15(**R**)16-*p*-Fluorophenoxy-17,18,19,20-tetranor $PGF_{2\alpha}$ (ICI

79939) was supplied by ICI Pharmaceuticals. 2(S),3(S),3″(S)2-(6′-carboxyhex-2′Z-enyl)-3-(3″-hydroxyoct - 1″E - enyl) - bicyclo [3,1,1]-heptane (Carbocyclic thromboxane A_2, CTA_2) was a gift from May & Baker Pharmaceuticals. PGA_2 (90%) was purchased from Medicuba Alida, Havana, Cuba and purified by us. The **R**(+)-isomer of trimethoquinol (TMQ) was a gift from the Tanabe Seiyaka Co. Ltd., Japan. The thromboxane synthetase inhibitor dazoxiben (UK 37248) was a gift from Pfizer UK, Sandwich, Kent. 3,3,4,4-Tetradeutero $PGF_{2\alpha}$ was purchased from Merck, Sharp & Dohme Canada Ltd.

Results

Examination of the radio-ligand

The [^{3}H]-15(S)9,11-epoxymethano PGH_2 ligand was compared with the authentic 'cold' compound for ability to aggregate human platelets and to contract the isolated dog saphenous vein. The nature of the responses (e.g. rates of onset and decline) for both samples was identical. The biological activity on the dog saphenous vein was used to estimate the amount of the ligand present (about 0.2 mg from each chromatographic separation) and hence to calculate the specific activity. The ligand was stable when stored in ethanol (1 mCi ml^{-1}) at −20°C; after 6 months no additional products could be detected by radio-scanning t.l.c.

Characterization of the binding of [^{3}H]-15(S)9,11-epoxymethano PGH_2 to washed human platelets

Time course Incubation of the washed platelet suspension with a fixed concentration of [^{3}H]-15(S)9,11-epoxymethano PGH_2(71 nM) at room temperature indicated considerable binding of the ligand by platelets. The amount bound reached a plateau within 2 min and remained stable for up to 4 min (Figure 2). Part of the bound label could be rapidly displaced by the addition of excess 'cold' 9,11-epoxymethano PGH_2 (11.4 μM). All subsequent incubations were of 4 min duration unless otherwise stated.

Non-displaceable binding In initial experiments the binding of [^{3}H]-15(S)9,11-epoxymethano PGH_2 was studied over the concentration range 71–607 nM. The dpm bound were converted into mol bound (a specific activity of 13.9 Ci mmol^{-1} is equivalent to 30.6 d min^{-1} fmol^{-1}) and divided by the platelet count to obtain the number of fmol bound per 10^6 platelets. Each concentration of the radioligand was tested in the presence and absence of a

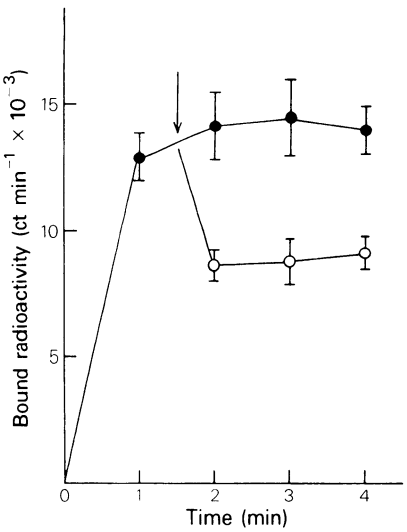

Figure 2 Time course of binding of [^{3}H]-15(S)9,11-epoxymethano PGH_2 (71 nM) to washed human platelets at room temperature (●). At the arrow, cold 15(S)9,11-epoxymethano PGH_2 (final concentration = 11.4 μM) was added to displace the radio-ligand (○). The values represent the mean and standard error of 12 observations on platelets from 2 donors.

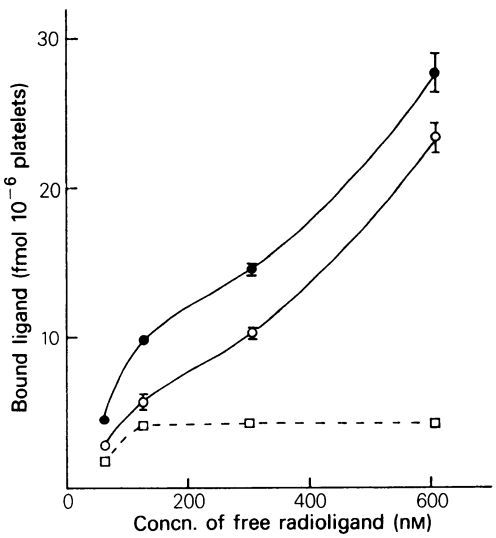

Figure 3 Dependence of [^{3}H]-15(S)9,11-epoxymethano PGH_2 binding on the free ligand concentration. Displaceable binding (□) was estimated by subtraction of non-displaceable binding (○) from total binding (●). Non-displaceable binding was measured in the presence of 11.4 μM cold 15(S)9,11-epoxymethano PGH_2. Each value is the mean of 18 observations on platelets from 3 donors; standard errors are shown for the total and non-displaceable binding only.

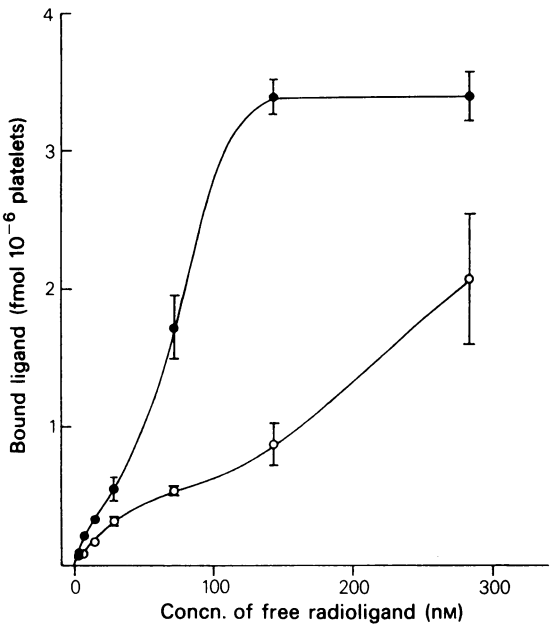

Figure 4 Displaceable binding for the [^{3}H]-15(**R**) (○) and [^{3}H]-15(**S**) (●) epimers of 9,11-epoxymethano PGH_2. The displacing agent was 15(**S**)9,11-epoxymethano PGH_2 (11.4 μM) in each case and displaceable binding was calculated as in Figure 3. Each point represents the mean of 20 observations on platelets from 5 donors. Standard errors derive from the 5 sub-means, and where error bars are not drawn, they fall within the diameter of the symbol.

high concentration of cold 9,11-epoxymethano PGH_2 (11.4 μM). Displaceable binding was determined by subtraction of non-displaceable binding from total binding (Figure 3). These data show that a large component of the binding is non-displaceable and linear in nature.

Nature of the displaceable binding It can be seen from Figure 3 that the amount of displaceable binding does not increase when the free ligand concentration is raised above 150 nM. Further experiments were carried out using lower concentrations of [^{3}H]-15(**S**)9,11-epoxymethano PGH_2 in order to characterize the displaceable binding (Figure 4). It is clear that the displaceable binding curve is not a simple hyperbola, and appears to have 2 components. When PGE_1 was omitted during the preparation of the platelet suspension the same binding profile was observed. The smaller component of binding is seen with low concentrations (1–20 nM) of the [^{3}H]-15(**S**) epimer and appears to account for about 15% of the total displaceable binding. The curve for the larger binding component appears to be sigmoidal in shape, perhaps indicating deviation from the simplest binding model (i.e. a single non-interacting population of binding sites). It is relevant that the concentration range corresponding to the larger binding component in Figure 4 is similar to the concentration range over which submaximal aggregation responses of human platelets to 15(**S**)9,11-epoxymethano PGH_2 are obtained. Thus in plasma at 37°C a shape change is recorded between 15 and 50 nM of the agonist and either a primary wave or a primary wave followed by a secondary wave between 100 and 250 nM.

The total number of displaceable binding sites is estimated to be about 2000 per platelet (calculated from data shown in Figures 3 and 4).

*Binding of [^{3}H]-15(**R**)9,11-epoxymethano PGH_2 to washed human platelets*

The displaceable binding curve obtained when [^{3}H]-15(**R**)9,11-epoxymethano PGH_2 is used as the radio-ligand and cold 15(**S**)9,11-epoxymethano PGH_2 as the displacing agent is shown in Figure 4. As with the 15(**S**) radio-ligand there is a small displaceable binding component at concentrations below 30 nM. With higher concentrations of the 15(**R**) radio-ligand the amount of displaceable binding is much less than that obtained with comparable concentrations of the 15(**S**) radio-ligand. In platelet-rich plasma 15(**R**)9,11-epoxymethano PGH_2 causes platelet shape change but never an aggregation wave (threshold concentration = 2 μM, highest concentration tested = 10 μM). Simultaneous addition of 15(**R**)9,11-epoxymethano PGH_2 and 11,9-epoxymethano PGH_2 (our standard full agonist in the aggregation studies) results in partial inhibition of the aggregatory action of the full agonist. These observations can be explained in terms of a partial agonist action of the 15(**R**) epimer; the weak blocking activity of the 15(**R**) epimer indicates a low affinity for the thromboxane receptor.

*Displacement of binding by the 15(**R**) and 15(**S**) epimers of PTA_2*

The possibility that platelets possess two different displaceable binding sites was investigated in greater detail using the [^{3}H]-15(**R**) and [^{3}H]-15(**S**) epimers of 9,11-epoxymethano PGH_2 as radio-ligands and the 15(**R**) and 15(**S**) epimers of PTA_2 as potential displacing agents. Both the latter compounds are enantiomerically pure and have the natural prostane configuration. 15(**S**) PTA_2 (absolute configuration at C15 identical to that of TXA_2) is a potent inhibitor of thromboxane-induced platelet aggregation (Table 1) whereas 15(**R**) PTA_2 is only about 1/40th as active.

The 15(**S**) PTA_2 epimer caused a maximum displacement of the 15(**S**) radio-ligand (71 nM) of 46%,

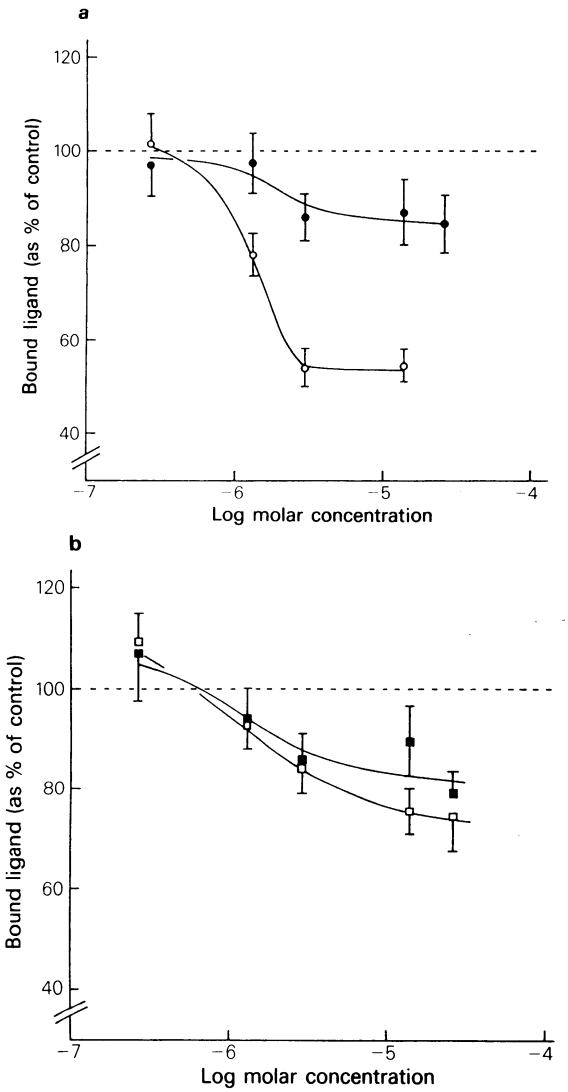

Figure 5(a) Displacement of [^{3}H]-15(S)9,11-epoxymethano PGH_2 (71 nM) by the 15(**R**) epimer (●) or 15(**S**) epimer (○) of PTA_2. Each value is the mean of 24 observations on platelets from 4 donors; s.e.mean shown by vertical lines. (b) Displacement of [^{3}H]-15(**R**)9,11-epoxymethano PGH_2 (71 nM) by the 15(**R**) epimer (■) or 15(**S**) epimer (□) of PTA_2. Each value is the mean of 12 observations on platelets from 2 donors; s.e. mean shown by vertical lines. Total binding in the absence of displacing agent = 100%.

comparable with that produced by cold 15(**S**)9,11-epoxymethano PGH_2 (Figure 5a). The 15(**R**) PTA_2 epimer gave a much smaller maximum displacement. With the 15(**R**) radio-ligand (71 nM) the major fraction of the binding was non-displaceable but the displacing activity of 15(**S**) PTA_2 was very similar to that of its 15(**R**) epimer (Figure 5b).

The data are compatible with the existence of 2 types of displaceable binding. With the 15(**S**) radio-ligand, the major component is displaced by 15(**S**) but not by 15(**R**) PTA_2 and we assume that this component represents the pharmacological receptor. The minor component is displaced by both 15(**R**) and 15(**S**) PTA_2. In contrast, using the 15(**R**) radio-ligand, the receptor-associated binding at 71 nM is small and the 15(**R**) and 15(**S**) PTA_2 displace the other component to a similar extent.

Displacement experiments with other compounds

A number of compounds were investigated for their ability to displace the [^{3}H]-15(**S**)9,11-epoxymethano PGH_2 ligand (71 nM) and the displacement curves are shown in Figure 6 and the results summarized in Table 1. All incubations were carried out at room temperature except in experiments with PGH_2 and TXA_2 where the temperature was 0°C. For compounds producing a maximum displacement similar in magnitude to the 15(**S**)9,11-epoxymethano PGH_2 analogue, the concentration corresponding to 25% displacement of total binding was calculated (Table 1). This group includes all the natural and synthetic bicyclic compounds tested, as well as ICI 79939, 16,16-dimethyl $PGF_{2\alpha}$ and arachidonic acid. Five compounds caused slight displacement which was always less than 20% at the highest concentration tested (Table 1). They were 15-methyl $PGF_{2\alpha}$, the 10α,11α-epoxide of PGA_2, PGD_2, PGE_1 and dazoxiben. **R**(+)-TMQ, PGE_2, $PGF_{2\alpha}$, EP 033 and oleic acid were devoid of displacing activity in concentrations up to 25, 14, 27, 150 and 700 μM respectively.

Aggregation experiments

The pro- and anti-aggregatory activities of the compounds used as displacing agents are shown in Table 1. For the stable bicyclic full agonists the data are reproduced from Wilson *et al.* (1982). The direct agonist activity is quantified by estimation from the log concentration-response curve of the concentration required for a primary aggregation wave of 20 units on the chart recorder; maximum aggregation gives a response of 80–90 units. Activities for the $PGF_{2\alpha}$ analogues, TXA_2, PGH_2 and arachidonic acid were calculated similarly.

With 9,11-ethano PGH_2 and CTA_2, only a rapidly reversing aggregation wave is seen, which with CTA_2 at the highest concentration tested (10 μM) has a magnitude of 15–20 units on the chart recorder. If a full agonist such as 11,9-epoxymethano PGH_2 is added simultaneously with either 9,11-ethano PGH_2 or CTA_2 then its aggregating activity is inhibited

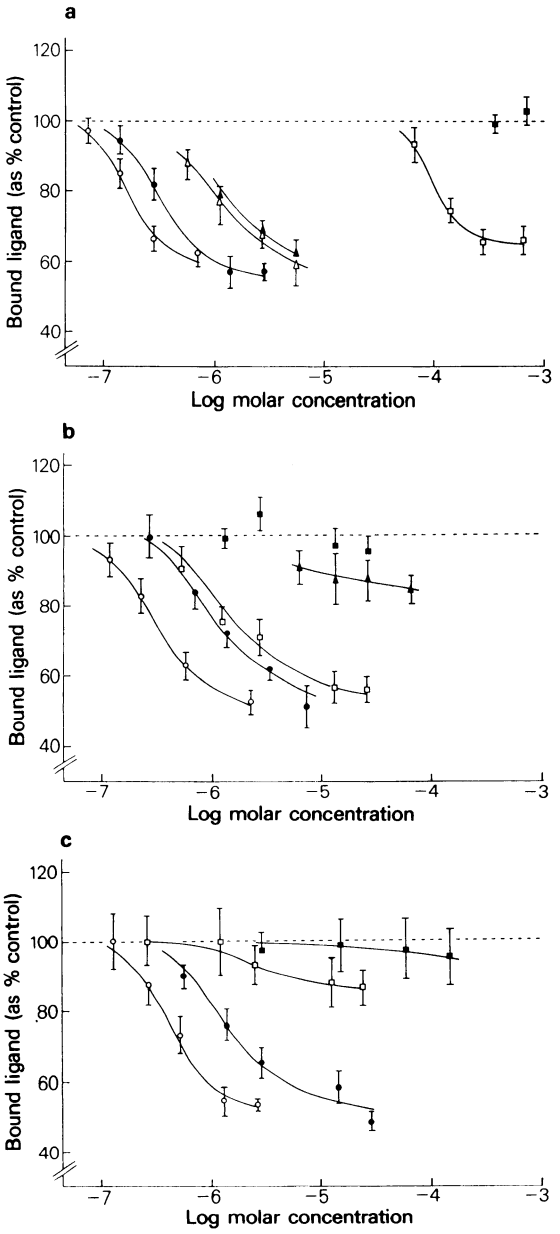

Figure 6 Displacement of [^{3}H]-15(**S**)9,11-epoxymethano PGH_2 (71 nM) binding by various compounds. In (a), 9,11-epoxymethano PGH_2 (○), 11,9-epoxymethano PGH_2 (●), TXA_2 (△), PGH_2 (▲), arachidonic acid (□) and oleic acid (■). In (b), ICI 79939 (○), 9,11-azo PGH_2 (●), 10a-homo-9,11-ethano PGH_2 (□), $PGF_{2\alpha}$ (■) and 15-methyl $PGF_{2\alpha}$ (▲). In (c), EP 045 (○), CTA_2 (●), dazoxiben (□) and EP 033 (■). Total binding in the absence of displacing agent = 100%. For each analogue all concentrations were tested on platelets from 3 different donors.

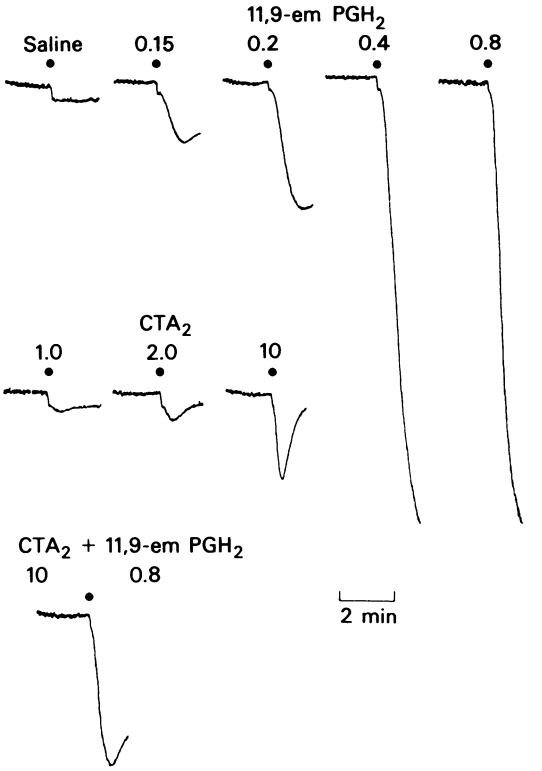

Figure 7 Aggregation of human platelets *in vitro*. 11,9-epoxymethano PGH_2 produces a reversible aggregation wave at low concentrations and irreversible aggregation at higher concentrations (top trace). CTA_2 produces a reversible wave only (middle trace) and opposes the action of a maximally aggregating dose of 11,9-epoxymethano PGH_2 added simultaneously (lower trace). Final concentrations (μM) are shown.

(Figure 7). However, substitution of ADP for 11,9-epoxymethano PGH_2 in these experiments results in an additive interaction between ADP and the bicyclic analogue.

For inhibitors of aggregation log dose-response curves for 11,9-epoxymethano PGH_2 were constructed in the presence of several concentrations of inhibitor. With all of the analogues examined, parallel shifts to the right of the agonist concentration-response curve were produced provided that the dose-ratio (DR) did not exceed 4. By estimating the concentration required for a DR of 2 it was possible to compare the inhibitory activities of all the compounds irrespective of their mechanism of action (Table 1). At concentrations of the inhibitors 10 fold higher than those given in Table 1 qualitative differences were seen. With the thromboxane specific group the block was still surmountable by increasing the agonist concentration. However, with inhibitors

Table 1 Comparison of radioligand displacement and biological activity on human platelets *in vitro*

Classification of biological action	*Compound*	*Conc. for 25% displacement of total binding (μM)*	*Conc. giving 20 unit aggregation response or Conc. for DR of 2.0 (μM)*
Aggregating agents			
Full agonists on the TX system, producing irreversible aggregation	9,11-epoxymethano PGH_2	0.20	0.26
	11,9-epoxymethano PGH_2	0.36	0.23
	9,11-azo PGH_2	1.1	0.050
	10a-homo-9,11-ethano PGH_2*	1.6	1.3
	EP 011*	1.2	0.19
	ICI 79939*	0.32	0.62
	16,16-dimethyl $PGF_{2\alpha}$	0.77	1.6
	15-methyl $PGF_{2\alpha}$	>68	22
	PGH_2	1.6†	0.38
	TXA_2 (PGH_2/HPM)	1.3†	0.27
	Arachidonic acid	140	180
Partial agonists on the TX system, producing reversible aggregation only: antagonism of full agonist action	9,11-ethano PGH_2*	0.44	15
	CTA_2	1.5	>10
Inhibitors of aggregation			
Compounds producing a selective block of thromboxane-like agonists: mechanism under investigation in this study.	EP 045*	0.46	1.3
	PTA_2	1.4	1.5
	R(+)–TMQ	>28	0.11
	$PGF_{2\alpha}$	>28	2.5
Compounds which inhibit all aggregating agents: known to raise cyclic AMP levels	PGE_1	>14	0.0038
	PGE_2	>14	1.5
	PGD_2	>14	0.020
	10α,11α-oxido PGA_2	>18	0.026

For all 15-hydroxy prostaglandin analogues, the orientation of the hydroxyl group relative to the carbon chain is the same as in TXA_2 (Figure 1); all compounds are designated 15(**S**) except EP 011, ICI 79939 and 16,16-dimethyl $PGF_{2\alpha}$ which have the 15(**R**) configuration.

*Indicates a racemic compound. †Binding study performed at 0°C.

Platelet aggregation data from Wilson, Peesapati, Jones & Hamilton (1982), Jones, Wilson, Armstrong, Peesapati & Smith (1983) and this study. Each value is the mean of at least 3 experiments. In the platelet aggregation inhibition studies 11,9-epoxymethano PGH_2 was used as the aggregating agent.

which act by raising platelet cyclic AMP levels (e.g. PGD_2) the aggregation response to even the highest concentration of 11,9-epoxymethano PGH_2 (10 μM) was completely inhibited.

Discussion

The analogue into which we have incorporated a tritium label, 15(**S**)9,11-epoxymethano PGH_2, produces TXA_2-like irreversible aggregation of human platelets *in vitro*. Its primary wave of aggregation is unaffected by the cyclo-oxygenase inhibitor, indomethacin, suggesting a direct agonist action on thromboxane receptors (Wilson *et al.*, 1982). From pharmacological studies on isolated smooth muscle preparations 15(**S**)9,11-epoxymethano PGH_2 has mimimal PGE_2-like or $PGF_{2\alpha}$-like activity (Jones, unpublished observations). The radio-ligand is enantiomerically pure, having been prepared from natural PGA_2, and is chemically stable.

Intact platelets were examined since centrifugation offers a rapid and reproducible means of separating bound and free liquid. Binding of [^{3}H]-15(**S**)9,11-epoxymethano PGH_2 reached a stable level within 1 min at room temperature; this finding agrees with the rapid onset of aggregation seen in platelet-rich plasma at 37°C (cf. Figure 7). Dissociation of the radio-ligand was also swift with a half-life of less than 0.5 min. Our standard incubation time of 4 min allows for attainment of equilibrium when other competing analogues with potentially slower kinetics are included in the system.

The total bound radioactivity appears to be the sum of 3 types of binding: (1) A non-displaceable linear component. This may reflect dissolution of the ligand in fatty regions of the platelet such as the plasma membrane. 9,11-Epoxymethano PGH_2 is a lipophilic compound and has a partition coefficient between chloroform and water (pH 7.4) of 19; the partition coefficient for $PGF_{2\alpha}$ under similar conditions is 0.009 (Jones, unpublished observations). We are attempting to prepare a radio-ligand with greater water solubility in the hope that it will have less non-displaceable binding. Considering the data presented in Table 1, the 16-*p*-fluorophenoxy-17,18,19,20-tetranor analogue of natural $PGF_{2\alpha}$ (ICI 79939 is the racemic form of this compound) is our first choice for introduction of a tritium label. However, it may be less useful than our present ligand since it is likely to have high affinity for $PGF_{2\alpha}$ receptors (see Dukes, Russell & Walpole, 1974, for $PGF_{2\alpha}$-like activity of ICI 79939). (2) A small displaceable binding component evident at low concentrations of both 15(**R**) and 15(**S**) radio-ligands. The experiments performed with the 15(**R**) and 15(**S**) epimers of PTA_2 also suggest a low specificity in relation to the C15 secondary alcohol for this binding component. We know of no biological activity demonstrable at concentrations of 15(**R**)9,11-epoxymethano PGH_2 below 100 nM, so the significance of this binding component is not clear. (3) A specific, displaceable binding component. This is the larger of the saturable components. The basis of the sigmoidal binding curve cannot be elucidated from the limited binding data we have. A more detailed experimental analysis is required to determine the nature of any interaction between binding sites (allosterism) (see Nichol, Jackson & Winzor, 1967). We can only characterize this specific binding system by stating that the number of 15(**S**)9,11-epoxymethano PGH_2 molecules bound per platelet is about 1700 (85% of 2000 sites derived from displaceable binding experiments), and that 50% of these sites are occupied when the 15(**S**)9,11-epoxymethano PGH_2 concentration is about 75 nM.

The large size of the non-displaceable component obviously affects the accuracy with which the displaceable component can be measured. In the displacement studies a concentration of radio-ligand of 71 nM was chosen to allow (a) an optimal ratio between displaceable and non-displaceable binding and (b) good radio-counting precision without the need to raise the concentrations of displacing agents to inordinately high levels.

It is our hypothesis that this binding component represents the pharmacological receptor. Biological activity on the human platelet is dependent on the orientation of the C15 secondary alcohol (see Wilson *et al.*, 1982) and the data presented here with both the 15(**R**)/15(**S**) radio-ligand epimers and 15(**R**)/15(**S**) PTA_2 epimers indicate that the larger displaceable binding component is also sensitive to the stereo-chemistry at C15. Unfortunately a mirror image form (unnatural prostane structure) of one of the enantiomeric compounds with high displacing ability (e.g. 15(**S**)9,11-epoxymethano PGH_2 or PTA_2) was not available for study. At present we are attempting chromatographic resolution of several of the racemic analogues so that the displacing abilities of the mirror image forms may be compared.

It can be seen from Table 1 that the potent full agonists on human platelets including the natural agents TXA_2 and PGH_2 all effectively displace the radio-ligand. Arachidonic acid, which aggregates human platelets at concentrations of 100–500 μM also displaces the radio-ligand at similar concentrations. In both instances the effect is presumably due to conversion of arachidonic acid to prostaglandin endoperoxides and/or TXA_2 by the platelet. Oleic acid is not a substrate for the cyclo-oxygenase system and does not aggregate human platelets; it does not displace the radio-ligand (Figure 6a).

The high displacing ability of the $PGF_{2\alpha}$ analogue, ICI 79939, is of particular interest to us since we have previously suggested (Jones & Marr, 1977) that its vasoconstrictor, bronchoconstrictor and platelet aggregation activities might be due to interaction with TXA_2 receptors in addition to its known activity on $PGF_{2\alpha}$ receptors. This suggestion was made cautiously since the structural similarity between ICI 79939 and TXA_2 or the prostaglandin endoperoxides is not striking (Figure 1). The results obtained in the present study would tend to support our original hypothesis. The 15-methyl analogue of $PGF_{2\alpha}$ which is a very weak aggregating agent shows little ability to displace the ligand. An exact correlation between the concentration required for displacement and the concentration required for a fixed aggregation response cannot be expected since the agonist effect is, according to classical receptor concepts, dependent on both the efficacy and the affinity of the compound under investigation. It would appear that two compounds in particular, 9,11-azo PGH_2 and the 16-*p*-fluorophenoxy derivative of 9,11-etheno PGH_2 (EP 011) are agonists with high efficacy.

The dicarba analogue of TXA_2, CTA_2 (Lefer, Smith, Araki, Smith, Aharony, Claremon, Magolda & Nicolaou, 1980) and the 9,11-ethano analogue of PGH_2 also readily displace the radio-ligand. In human PRP these compounds produce only a primary wave of aggregation. When added simultaneously with 11,9-epoxymethano PGH_2 they oppose its aggregatory activity but act additively or synergistically with ADP. These actions are consistent with a reasonably high affinity for the thromboxane receptor coupled with a low efficacy, that is, they behave as partial agonists.

Both PTA_2 and EP 045 in concentrations up to 20 μM show no agonist activity on human platelets. They oppose the aggregatory action of 11,9-epoxymethano PGH_2 but not that of ADP. The displacement of the radio-ligand by these compounds strongly suggests that their inhibitory action is due to occupation of thromboxane receptors, that is, they are thromboxane receptor antagonists. Other studies (Jones *et al.*, 1983) have shown that EP 045 does not raise cyclic AMP levels and thus a contribution to its inhibitory action through activation of PGI_2 or PGD_2 receptors seems unlikely. Removal of the phenyl group from EP 045 results in a compound, EP 033, which has weak anti-aggregatory activity (dose ratio at 20 μM is less than 2.0) and little ability to displace the radio-ligand (Figure 6c).

$PGF_{2\alpha}$ also inhibits 11,9-epoxymethano PGH_2 but not ADP aggregation responses. This action of $PGF_{2\alpha}$ was only recently demonstrated by Hung, Ghali, Ventan & Le Breton (1982). They showed that [^{3}H]-$PGF_{2\alpha}$ was bound to human isolated platelet membranes and could be displaced by 11,9-epoxymethano PGH_2 and 13-azaprostanoic acid but not by thromboxane B_2 or 6-keto $PGF_{1\alpha}$. They concluded that $PGF_{2\alpha}$ interacts directly with the TXA_2/PGH_2 receptor to produce its inhibitory action. Our ligand binding results would tend to suggest that this is not the case and that an alternative mode of action for $PGF_{2\alpha}$ must be sought.

Trimethoquinol is structurally unrelated to the prostanoid group of compounds (Figure 1). The **R**(+)-isomer exhibits potent anti-aggregatory effects against 9,11- and 11,9-epoxymethano PGH_2, arachidonic acid, and collagen but not against ADP (primary wave). The **S**(−)-isomer is much less active on aggregation but exhibits β-adrenoceptor agonist activity (Akbar, Navran, Miller & Feller, 1981; Mayo, Navran, Akbar, Miller & Feller, 1981). In our experiments **R**(+)-TMQ inhibited aggregation due to 11,9-epoxymethano PGH_2 in the concentration range 0.1 to 1 μM. Displacement of the radio- ligand was not observed with concentrations of up 28 μM. It is unlikely therefore that **R**(+)-TMQ behaves as a thromboxane receptor antagonist on human platelets, and further experiments into its mechanism of action are in progress.

PGE_1, PGD_2 and the 10α,11α-oxide of PGA_2, are potent inhibitors of platelet aggregation induced by TX-like agents, ADP, thrombin and collagen, and exert their effects by raising cyclic AMP levels within the platelet. None of these compounds caused a reduction in binding of the 15(**S**) radio-ligand. PGE_2, which at low concentrations (20–100 nM) potentiates second phase aggregation (Shio & Ramwell, 1972; Willis, Vane, Kuhn, Scott & Petrin, 1974) and at high concentrations (1–10 μM) in platelet samples from some donors inhibits aggregation (Chandrasekhar, 1970; these studies), was also ineffective as a displacing agent.

There has been some speculation as to the existence of receptors for the prostaglandin endoperoxides distinct from those at which TXA_2 is the most active natural agonist (Needleman, Minkes & Raz, 1976; MacIntyre & Gordon, 1977). It is important to note that no results have yet been presented in which PGG_2 (or PGH_2) is shown to be consistently more active than TXA_2 and where spontaneous or enzymatic conversion of the endoperoxides to other biologically active compounds (e.g. PGD_2, PGE_2 or $PGF_{2\alpha}$) has been conclusively ruled out. It is undoubtedly true that thromboxane-sensitive smooth muscle preparations (TXA_2 more active than PGG_2/PGH_2) such as the rabbit isolated aorta show quite different agonist sensitivities from those found for human platelets. For example, CTA_2 is a very potent agonist on the perfused cat coronary artery (Lefer *et al.*, 1980) and is a full agonist on the rabbit aorta, dog saphenous vein and guinea-pig trachea (Jones, unpublished observations). PTA_2 and 9,11-

epoxymethano PGH_2 are partial agonists on the latter 3 preparations (Jones *et al.*, 1982). In contrast, 9,11-epoxymethano PGH_2 is a full agonist, CTA_2 a partial agonist (these studies) and PTA_2 an antagonist (Nicolaou *et al.*, 1979) on human platelets *in vitro*. Our binding data show that compounds with ring systems, related to either the prostaglandin endoperoxides (*bicyclo* [2,2,1] heptane) or thromboxane A_2 (*bicyclo* [3,1,1] heptane) show high affinity for the major displaceable site, complementing the biological results obtained from aggregometry studies. Of particular interest would be a comparison of the aggregatory potencies and the binding affinities of the mono-thia and the mono-carba analogues of TXA_2 (CTA_2 is the dicarba analogue of TXA_2).

We prefer to call the human platelet receptor a thromboxane receptor, reflecting the slightly greater agonist potency of TXA_2 relative to PGH_2. However, we do recognise that *in vivo* both the prostaglandin endoperoxides and TXA_2 may exert simultaneous agonist activities at this receptor. As far as vascular or bronchial smooth muscle is concerned, TXA_2 may be the more important agent by virtue of its greater activity. Differences in agonist potency between two closely related natural ligands as a result of the existence of two or more receptor subtypes is well documented. For example, noradrenaline and adrenaline have similar agonist activities on the β_1-adrenoceptor but adrenaline is much more active on the β_2-adrenoceptor. We hope to extend our ligand binding studies and compare the properties of thromboxane receptors in smooth muscle cells with those in the blood platelet.

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