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Prostaglandin endoperoxide analogues which are both thromboxane receptor antagonists and prostacyclin mimetics

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1 Two prostaglandin endoperoxide analogues, EP 035 and EP 157, behave as specific thromboxane receptor antagonists on isolated smooth muscle preparations such as rabbit aorta, dog saphenous vein and guinea-pig trachea. However, in human platelet-rich plasma (PRP) they produce an unsurmountable block of aggregation induced by a wide range of agents (ADP, platelet-activating factor, thrombin); this inhibitory profile is typical of that seen with either prostaglandin I_2 (PGI_2) or PGD_2.

2 EP 035 and EP 157 induce large increases in cyclic AMP levels (up to 20 times basal) in human PRP. Simultaneous exposure to PGE_1 markedly reduces their effect on cyclic AMP; exposure to PGD_2 is much less effective in this respect. The adenylate cyclase inhibitor SQ 22,536 opposes the inhibitory action of EP 035, EP 157, iloprost (a stable PGI_2 analogue) and PGD_2 on platelet aggregation. However, the xanthone derivative AH 6809 blocks the inhibitory action of PGD_2 but does not affect EP 035, EP 157 and PGI_2 and its structural analogues.

3 EP 035 and EP 157 displace [^{3}H]-iloprost binding to the PGI_2 receptor on human platelet membranes. Displacing ability is ranked as follows: iloprost > 6a-carba PGI_2 > EP 157 > EP 035 > EP 164 (α-dinor derivative of EP 157). This order of potency is the same as that found for activation of adenylate cyclase in homogenates of washed human platelets and for inhibition of aggregation in washed human platelets.

4 The activities of EP 035 and EP 157 were studied in two other systems containing PGI_2 receptor-adenylate cyclase complexes, the NCB-20 cell line and human lung tissue. In both cases stimulation of adenylate cyclase was found but maximum rates were below that achieved with iloprost. These effects of EP 035 and EP 157 could be correlated with their abilities to displace [^{3}H]-iloprost binding.

5 These results indicate that EP 035 and EP 157 inhibit the aggregation of human platelets by acting as agonists at the PGI_2 receptor linked to adenylate cyclase. They represent a class of compound with both thromboxane receptor blocking activity and prostacyclin mimetic activity.

Introduction

Thromboxane A_2 (TXA_2) and prostaglandin I_2 (PGI_2, prostacyclin), both formed enzymatically from a common precursor PGH_2, have opposing actions in several biological systems. For example, TXA_2 produces vasoconstriction and aggregation of blood platelets (Hamberg *et al.*, 1975) whereas PGI_2 produces vasodilatation and inhibition of platelet aggregation (Moncada *et al.*, 1976). From a therapeutic standpoint the moderation of thromboxane-induced events with no effect or even enhancement of prostacyclin-induced events may be beneficial in certain cardiovascular disorders. This could be achieved through the use of thromboxane synthesase inhibitors, thromboxane receptor antagonists or stable PGI_2 mimetics. We have concentrated our efforts on the development of high affinity thromboxane receptor antagonists. Two analogues, EP 045 and EP 092 (Figure 1) are typical of the series. They are prostanoid in nature with a ring system formally related to PGH_2. They specifically block the stimulant actions of TXA_2 and its stable mimetics on both vascular smooth muscle and platelets (Jones *et al.*, 1982; Armstrong *et al.*, 1985a). Primary aggregation waves induced in human platelet-rich plasma (PRP)

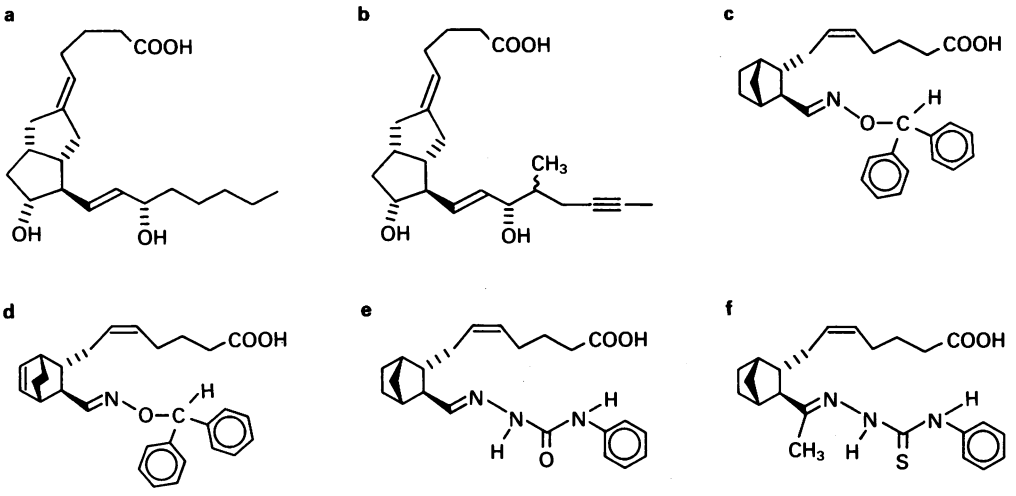

Figure 1 Chemical structures of (a) 6a-carba prostaglandin I_2, (b) iloprost (ZK 36,374), (c) EP 035, (d) EP 157, (e) EP 045 and (f) EP 092. The four EP analogues are racemic.

by adenosine 5'-pyrophosphate (ADP), adrenaline and platelet-activating factor (Paf) are not inhibited by EP 045 and EP 092, and this correlates with their inability to raise adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels.

We have however identified several analogues, each with a diphenylmethyl group in the ω-chain, which show a medium degree of thromboxane receptor block on several smooth muscle preparations but a very different profile of inhibitory activity on human platelets. The structures of two of these analogues, EP 035 and EP 157, are shown in Figure 1. EP 035 and EP 157 completely inhibit aggregation responses induced by supra-maximal concentrations of thromboxane mimetics, ADP, adrenaline and Paf, and this is accompanied by significant rises in cyclic AMP levels. This behaviour is typical of PGI_2 and PGD_2 (Tateson *et al.*, 1977) but the structural relationship between EP 035/EP 157 and the natural prostanoids is not obvious. This paper describes our efforts to elucidate the mechanism of action of the two novel analogues.

Methods

Experiments on intact human platelets

Platelet aggregation Human blood was freshly collected by venepuncture. Platelet-rich plasma (PRP) and washed platelet suspensions were prepared as described previously (Armstrong *et al.*, 1985a). Photometric measurements of platelet aggregation were made. A polystyrene cuvette containing 3 ml of platelet suspension was contained in a thermostatic cell block (37°C), and stirring was achieved with a stainless steel rod revolving at 1000 r.p.m. Potential inhibitors were added to the cuvette 2 min before the aggregating agent.

Cyclic AMP determinations PRP in 1 ml aliquots was incubated with selected concentrations of prostaglandin or saline control for 30 s at 37°C, and the reaction quenched by the addition of ethanol. The samples were extracted and the cyclic AMP levels measured by a protein-binding assay as described previously (Armstong *et al.*, 1985a). For examination of washed platelets, the PRP was further centrifuged at 400 *g* for 15 min and the pellet resuspended in saline. No PGI_2 was employed. Cyclic AMP levels were expressed as multiples of the basal level.

Displacement of [^{3}H]-9,11-epoxymethano PGH_2 binding Displacement of [^{3}H]-9,11-epoxymethano PGH_2 (71 nM, specific activity 14 Ci mmol^{-1}) from washed human platelets was measured as described previously (Armstrong *et al.*, 1983). Bound and free radioligand were separated by rapid centrifugation after incubation for 4 min at room temperature.

Experiments on homogenates and membrane preparations

Human platelets Platelet homogenates were employed in the measurement of adenylate cyclase activity. They were prepared from the original platelet pellet by homogenisation in 25 mM Tris-HCl buffer pH 7.4 containing 0.29 M sucrose with 20 strokes of a tightly fitting dounce homogeniser.

Platelet membranes were prepared from platelet

concentrate packs kindly supplied by the Blood Transfusion Service, Royal Infirmary, Edinburgh. After dilution with an equal volume of 50 mM Tris pH 7.4, the platelet suspension was centrifuged at 400 *g* for 15 min. The pellet was resuspended in 5 mM Tris pH 7.4 and homogenised before being centrifuged at 100,000 *g* for 15 min at 4°C. This procedure was repeated and the membrane pellet finally suspended in 50 mM Tris pH 7.4. Protein concentration was determined by the method of Lowry *et al.* (1951) and adjusted to 4 mg ml^{-1}.

NCB-20 cells NCB-20 cells were cultured and homogenates and membrane suspensions prepared as described previously (Blair & MacDermot, 1981). The protein concentration of the membrane suspension was adjusted to 1 mg ml^{-1}.

Human lung Human lung tissue was obtained from a lobectomy specimen (Colindale Hospital, London). Surgery in this non-smoker was for a carcinoma. The tissue was chopped, minced and homogenised in 25 mM Tris buffer pH 7.4 containing 0.29 M sucrose (10 ml g^{-1}) using a Polytron. After filtration through nylon gauze, undisrupted cells and nuclei were removed by centrifugation at 500 *g* for 15 min at 4°C. The supernatant was retained and the pellet resuspended in buffer and centrifuged as before. Both supernatant fractions were pooled and used in the adenylate cyclase assay as tissue homogenate. A portion was also centrifuged at 100,000 *g* for 15 min at 4°C. The membrane pellet was suspended in 50 mM Tris pH 7.4 and the protein concentration adjusted to 3.75 mg ml^{-1}.

Adenylate cyclase measurements Enzyme activity in the homogenate preparations was measured as described previously (Leigh *et al.*, 1984). Reaction mixtures of 100 µl contained 50 mM Tris-HCl buffer pH 8.5; 5 mM magnesium chloride; 87 mM sucrose; 20 mM creatinine phosphate, disodium salt (Sigma); 10 International Units creatinine kinase, 150 iu mg^{-1} protein (ATP: creatinine N-phosphotransferase; EC 2.7.3.2) from Sigma; 1 mM cyclic adenosine 3':5'-monophosphate, sodium salt (Sigma); 0.25 mM Ro20-1724 (a phosphodiesterase inhibitor from Roche Products Ltd); 0.5% ethanol; 1 mM [^{32}P]-adenosine 5'-triphosphate ([^{32}P]-ATP; 3 µCi, Amersham Radiochemical Centre); and 100 to 200 µg of homogenate protein.

Displacement of [^{3}H]-iloprost binding The binding of [^{3}H]-iloprost (specific activity 19.5 Ci $mmol^{-1}$) to the three membrane preparations was studied. Each assay tube contained 2 pmol [^{3}H]-iloprost, 1 µmol $MgSO_4$, 5 µmol Tris-HCl pH 7.4 and cell membranes (human platelet 0.16 mg, NCB-20 cell 0.044 mg and human lung 0.15 mg) in a total volume of 100 µl. Prostaglandins were dissolved in part of the Tris buffer with the aid of ethanol (final concentration = 1%). After incubation for 20 min at 30°C, bound radioligand was separated by rapid filtration through Whatman GF/B glass filter discs. Each filter was washed twice with 4 ml 50 mM Tris buffer pH 7.4, dried and then suspended in Insta-Gel (Packard Instrument Co. Inc.) for scintillation counting.

Isolated smooth muscle preparations

Spiral strips of rabbit aorta, dog saphenous vein and guinea-pig trachea were set up in conventional organ baths (8 ml) for isometric tension recording as described previously (Jones *et al.*, 1982). Reproducible cumulative dose-response relationships were obtained for U-46619. The preparation was then exposed to the antagonist for 60 min (continuous perfusion of the organ bath with complete replacement every 2 min). The flow of antagonist solution was stopped and a new agonist dose-response relationship was obtained. Dose-ratios were calculated.

Compounds

EP 035, EP 045, EP 092, EP 157 and EP 164 were prepared in our laboratory. Stock solutions were prepared by adding an equimolar amount of sodium hydroxide to the free acid dissolved in ethanol, evaporating the ethanol, and dissolving the residue in 0.9% saline. PGI_2, iloprost and its tritium-labelled derivative were gifts from Schering AG, Berlin. 6a-Carba PGI_2 was a gift from Wellcome Research, Beckenham. U-46619 (11,9-epoxymethano PGH_2), PGE_1 and PGD_2 were purchased from Upjohn Diagnostics Ltd, U.S.A. AH 6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) was a gift from Glaxo Group Research, U.K. and SQ 22,536 [(9-tetrahydro-2-furyl) adenine] was a gift from Squibb Ltd., U.S.A.

Results

The pharmacological profile of EP 035 and EP 157

Thromboxane block on isolated smooth muscle preparations EP 035 and EP 157 at 10 µM completely blocked contractile responses (up to 80% of the maximum) induced by the thromboxane mimetic U-46619 (11,9-epoxymethano PGH_2) on the rabbit aorta, dog saphenous vein and guinea-pig trachea. In contrast, submaximal contractile responses to noradrenaline on the rabbit aorta, clonidine on the dog saphenous vein and histamine on the guinea-pig trachea were unaffected. 16,16-dimethyl PGE_2 contracts the guinea-pig trachea by activating specific PGE_2 receptors (Jones *et al.*, 1982). EP 035 and EP 157 at 10 µM did not block this action.

Table 1 Antagonism by EP 035 and EP 157 of the contractile action of U-46619 on isolated smooth muscle preparations

Antagonist	*U-46619 dose-ratio*		
	Rabbit aorta	*Dog saphenous vein*	*Guinea-pig trachea**
EP 035			
2.5 μM	2.8,3.1,6.0	19,36,44,53	5.4,6.9,8.2
10 μM	19,28,32		
EP 157			
2.5 μM	3.0,3.2,5.0	21,28,36	5.1,6.5,8.0
10 μM	13,17,22		

Each preparation was exposed to the antagonist for 60 min before the agonist was re-added. *Indomethacin (1×10^{-6} M) and atropine (2×10^{-8} M) were present in the Krebs solution bathing the guinea-pig trachea.

As a preliminary to determining affinity constants for EP 035 and EP 157 by the Schild procedure, the rate of attainment of equilibrium block was investigated. Since responses to U-46619 are not subject to fade, the most convenient method is to establish a submaximal response to a single dose of the agonist and then to observe the decay of tension following addition of the antagonist. EP 035 and EP 157 (e.g. at 0.5 μM on the dog saphenous vein) produced a slowly developing antagonism, much slower than that seen with roughly equi-effective concentratioins of EP 045 (0.25 μM) and EP 092 (0.05 μM). Tension continued to fall slowly even after 90 min contact. Although longer contact times are technically feasible, problems arise from changes in agonist sensitivity unrelated to the presence of the antagonist. In view of these difficulties it was decided to abandon the construction of Schild plots and simply determine dose-ratios after 60 min exposure (our usual contact time). These are given in Table 1. In all cases the rightward shift of the agonist log concentration-response curve was parallel in nature, as judged by eye. Reversal of the blocking action of EP 035 and EP 157 by continuous washing of the preparations was also very slow.

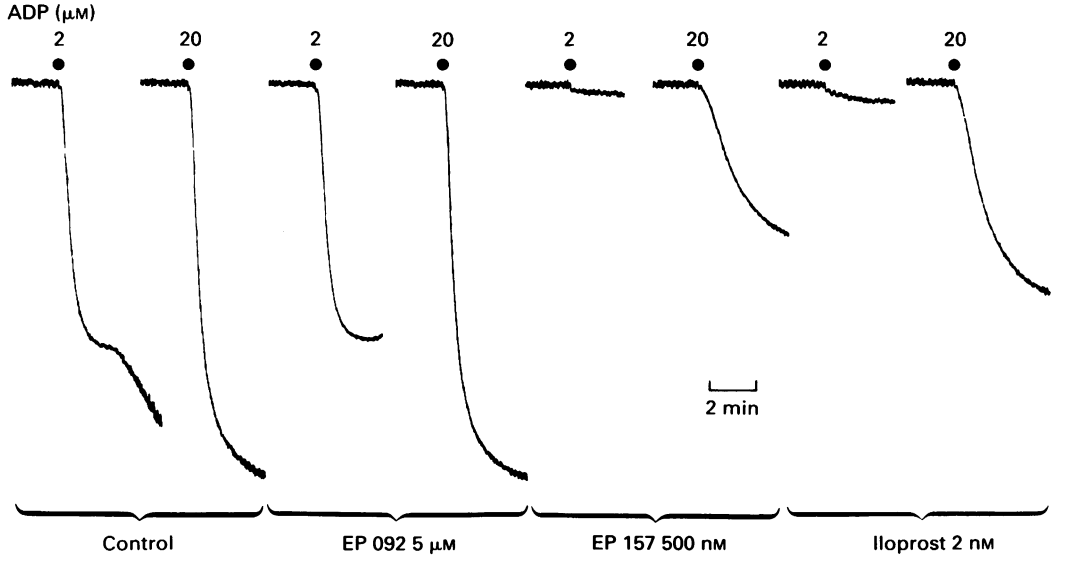

Figure 2 Human platelet-rich plasma: light transmission records. ADP was used as the aggregating agent. Preincubation for 2 min with a high concentration of the thromboxane receptor antagonist EP 092 only blocks the secondary phase of aggregation and does not affect irreversible aggregation induced by supramaximal concentrations of ADP. In contrast EP 157 and iloprost (a stable PGI_2 analogue) inhibit primary aggregation and suppress supramaximal responses.

Inhibition of platelet aggregation In human PRP, both EP 035 (5 μM) and EP 157 (0.5 μM) completely inhibited aggregation induced by ADP (2 μM), thrombin (2 μg ml^{-1}), Paf (0.1 μM), arachidonic acid (500 μM) and U-46619 (0.3 μM). This profile of activity is similar to that found with PGI_2 and PGD_2 and their structural analogues, but quite different from that seen with the specific thromboxane receptor antagonist EP 092. The latter compound antagonizes only the secondary wave of aggregation associated with the biosynthesis of prostaglandin endoperoxides and TXA_2 (Figure 2).

With low concentrations of EP 157 (e.g. 50 nM when ADP was the aggregating agent) a parallel rightward displacement of the log concentration-response curve for each aggregating agent was produced; at higher concentrations of EP 157 (250 nM) the curve was flattened such that only reversible aggregation could be induced. At even higher concentrations (1 μM) aggregation was completely inhibited irrespective of the dose of aggregating agent added. This unsurmountable inhibition is again typical of PGI_2 and PGD_2. EP 035 at concentrations ten fold higher than those of EP 157 behaved similarly. However, EP 035 and EP 157 do not inhibit the effects of each aggregating agent to exactly the same degree. The order of susceptibility to the inhibitory effect is U-46619: Paf: thrombin: ADP. Typically about one third as much of the EP analogue is required for 50% inhibition of U-46619-induced aggregation as compared to ADP. The same order of susceptibility to inhibition is also found for PGI_2 and PGD_2, and their mimetics (Armstrong *et al.*, 1985b). We could find no evidence that EP 035 and EP 157 exert a relatively greater inhibitory action against thromboxane-like aggregation than do PGI_2 and PGD_2.

SQ 22,536 is an inhibitor of the enzyme adenylate cyclase (Harris *et al.*, 1979). At 100 μM, SQ 22,536 partly reversed the inhibition of platelet aggregation produced by EP 035 and EP 157. It affected the inhibitory action of PGI_2, PGD_2 and PGE_1 in a similar manner. In contrast SQ 22,536 had no effect on the inhibition of aggregation mediated by the thromboxane receptor antagonists EP 045 and EP 092, as this is not accompanied by stimulation of adenylate cyclase

In human PRP, AH 6809 counteracts PGD_2-induced inhibition of aggregation whereas the action of PGI_2 is slightly enhanced (Keery & Lumley, 1985). Using ADP as the aggregating agent in the presence of indomethacin (10 μM), AH 6809 at 100 μM displaced the log concentration-response curve for PGD_2 (IC_{50} = 34 nM) to the right. Dose-ratios (DR) of 13.5, 13.9 and 14.5 were obtained in 3 separate experiments. In each case the inhibitory actions of EP 035 (IC_{50} = 900 nM), EP 157 (210 nM) and PGI_2 (1.8 nM) were slightly potentiated (DR = 0.78–0.90).

Accumulation of cyclic AMP in human PRP and interaction with PGE_1 and PGD_2 Addition of either EP 035 or EP 157 to human PRP induced large increases in platelet cyclic AMP levels. Concentration-response relationships are shown in Figure 3. Simultaneous exposure of the platelet suspension to PGE_1 (2.5 μM) markedly reduced the effect of EP 035 and EP 157 on cyclic AMP accumulation. PGD_2 (2.5 μM) was considerably less effective in this respect. The effects of exposure to PGE_1 and PGD_2 on cyclic AMP accumulation by the stable PGI_2 mimetic, iloprost are also shown in Figure 3.

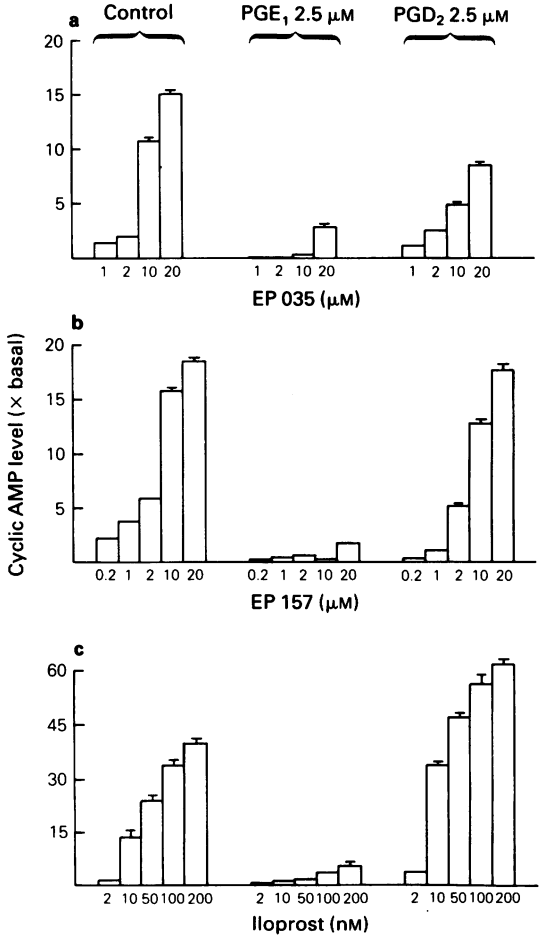

Figure 3 Elevation of cyclic AMP levels in human platelet-rich plasma induced by (a) EP 035, (b) EP 157 and (c) iloprost (control histograms) and the effects of simultaneous exposure of the platelets to prostaglandin E_1 (PGE_1) and PGD_2. Mean values, with vertical lines indicting s.e. mean, for 12 determinations (2 donors) are shown.

Overall these results suggest that EP 035 and EP 157 inhibit platelet aggregation by acting at the PGI_2 receptor linked to adenylate cyclase. The following experiments were designed first to substantiate the above hypothesis and secondly to measure the potency of EP 035 and EP 157 relative to stable PGI_2 analogues on systems where reduction of activity due to extraneous protein binding is minimal.

Experiments with washed platelet preparations

Inhibition of aggregation In the presence of indomethacin (1 μM), EP 035 and EP 157 inhibited irreversible aggregation waves in washed human platelet suspensions induced by ADP, Paf and U-46619. Unfortunately, responsiveness to ADP and Paf often declined during the course of the experiment. However, responses induced by U-46619 (0.1–0.2 μM) remained constant with time, allowing accurate comparisons of inhibitory activity using this aggregating agent. Figure 4 shows typical inhibition curves for iloprost, 6a-carba PGI_2, EP 035 and EP 157. Iloprost was used as the standard agonist (equipotent molar ratio: EPMR = 1) and its IC_{50} ranged from 0.064 to 0.18 nM ($n = 9$), reflecting its very high potency in this system. EPMRs (means ± s.e.mean) were calculated as follows: 6a-carba PGI_2 25 ± 5.9 ($n = 4$), EP 157 49 ± 12 ($n = 6$), EP 035 276 ± 26 ($n = 4$). EP 164, the α-dinor derivative of EP 157, showed only weak inhibitory activity (EPMR 6650 ± 330, $n = 3$).

Displacement of [^{3}H]-9,11-epoxymethano PGH_2 binding The saturable, displaceable component of the binding of [^{3}H]-9,11-epoxymethano PGH_2 (71 nM) to washed human platelets comprised about 50% of the total binding. EP 035 and EP 157 displaced saturable binding in concentrations in excess of 0.5 μM (Figure 4). These concentrations are at least 300 fold higher than those used in the aggregation experiments and this provides additional evidence that thromboxane receptor block does not make a significnt contribution to the inhibitory action of EP 035 and EP 157.

Activation of adenylate cyclase Activation of adenylate cyclase in human platelet homogenates by increasing concentrations of iloprost, 6a-carba PGI_2, EP 157 and EP 035 is shown in Figure 4. Concentrations of each compound producing an initial velocity of 20% of the maximum velocity obtained with iloprost (10 μM) are given in Table 2. The maximum velocity for EP 157 was similar to that for iloprost whereas 6a-carba PGI_2 and EP 035 gave slightly lower values. PGD_2 gave a maximum velocity equal to 34% of the iloprost value.

Displacement of [^{3}H]-iloprost binding The displacement of [^{3}H]-iloprost binding to human platelet membranes by iloprost, 6a-carba PGI_2, EP 157 and EP 035 is shown in Figure 4. IC_{50} values are given in Table 2. PGD_2 did not displace the radioligand at concentrations up to 10 μM. In addition, EP 045, EP 092 and EP 164 showed little ability to displace the radioligand; at 10 μM of each antagonist, bound radioligand was still 84, 91 and 90% of the control value.

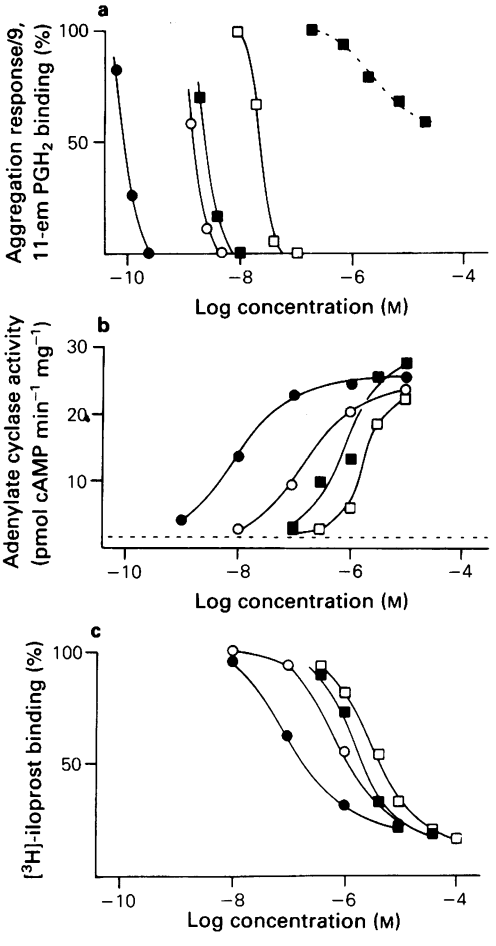

Figure 4 Protein-free human platelet preparations: log concentration-response relationships for iloprost (●), 6a-carba prostaglandin I_2 (6a-carba PGI_2; ○), EP 157 (■) and EP 035 (□) in single experiments. (a) Washed platelet suspension – inhibition of the irreversible aggregation response produced by U-46619 (solid lines). Indomethacin (1 μM) was present. The dashed line indicates displacement of [^{3}H]-9,11-epoxymethano PGH_2 (9,11-em PGH_2) binding to washed platelets by EP 157 (means of 6 replicates). (b) Activation of adenylate cyclase in platelet homogenates (means of 3 replicates). The basal activity is indicated by the dashed line. (c) Displacement of [^{3}H]-iloprost binding to platelet membranes (means of 3 replicates).

Table 2 Activation of adenylate cyclase and displacement of [^{3}H]-iloprost binding by selected prostanoids

Prostanoid	*Human platelet*	*NCB-20 cell line*	*Human lung*
	Adenylate cyclase activity in homogenates: concentration (nM) giving 20% of iloprost maximum velocity		
Iloprost	2.4	12	15
6a-carba PGI_2	50 (89%)	95 (78%)	200 (77%)
EP 157	290 (99%)	1,660 (49%)	790 (72%)
EP 035	1,050 (79%)	7,240 (24%)	2,090 (49%)
PGD_2	3,100 (34%)	—	—
	Displacement of [^{3}H]-iloprost binding to membranes: IC_{50} (nM)		
Iloprost	110	100	210
6a-carba PGI_2	870	210	600
EP 157	2,030	5,750	11,200
EP 035	4,532	18,600	>20,000
PGD_2	>10,000	—	—

All values are the means from three separate experiments. In the adenylate cyclase section values in parentheses refer to the percentage of the maximal iloprost activity achieved with 10 μM of the appropriate analogue.

Experiments with NCB-20 neuroblastoma cells

The PGI_2 receptor in NCB-20 neuroblastoma cells has been well characterized in terms of its interaction with prostanoid ligands (Leigh *et al.*, 1984; Hall & Strange, 1984). In particular PGD_2 (up to 10 μM) does not displace [^{3}H]-iloprost binding or activate adenylate cyclase. In contrast, EP 035 and EP 157 both displaced the radioligand and activated adenylate cyclase (Figure 5 and Table 2). At the highest concentration tested (10 μM), 6a-carba PGI_2, EP 157 and EP 035 had significantly lower activities than iloprost.

Experiments with human lung preparations

Iloprost, 6a-carba PGI_2, EP 157 and EP 035 all activated adenylate cyclase in human lung homogenates (Figure 5 and Table 2). As with the NCB-20 cell homogenates the latter three compounds at 10 μM were unable to match the iloprost maximal velocity.

Our attempts to measure the displacement of [^{3}H]-iloprost binding to human lung membranes were less than ideal due to the high non-specific binding (60% of total binding) of the radioligand. Nevertheless, iloprost, 6a-carba PGI_2, EP 157 and EP 035 exhibited displacement with the same rank order of potency as seen with the human platelet and NCB-20 cell membranes (Table 2).

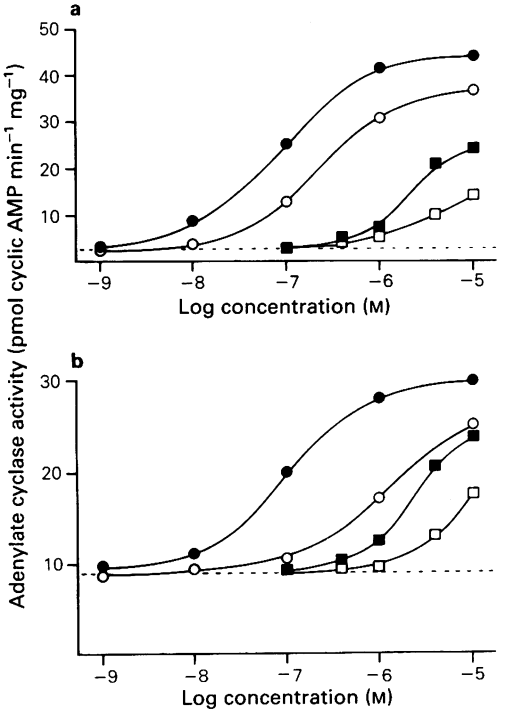

Figure 5 Activation of adenylate cyclase in homogenates of (a) NCB-20 neuroblastoma cells and (b) human lung tissue by iloprost (●), 6a-carba prostaglandin I_2 (6a-carba PGI_2; ○), EP 157 (■) and EP 035 (□). Each point is the mean of 3 replicates in a single experiment.

Discussion

The results obtained in this study strongly suggest that EP 035 and EP 157 act as functional antagonists on the human platelet and that the intermediary of their

inhibitory action is cyclic AMP. Thus EP 035 and EP 157 produce large rises in cyclic AMP levels in both PRP and washed platelet suspensions, and maximally or near maximally activate adenylate cyclase in platelet homogenates. In addition, the adenylate cyclase inhibitor SQ 22,536 reduces the inhibitory effect of both compounds on platelet aggregation.

Furthermore, four lines of evidence indicate that EP 035 and EP 157 activate adenylate cyclase by acting as agonists at PGI_2 receptors on the platelet membrane. This evidence is as follows: (1) the characteristics of the inhibition of platelet aggregation produced by EP 035 and EP 157 are very similar to those seen with PGI_2 and its stable (carba) analogues. (2) PGE_1 at high concentrations markedly suppresses the cyclic AMP stimulant action of EP 035 and EP 157; the same effect is achieved against iloprost. In contrast high concentrations of PGD_2 are substantially less effective. PGE_1 is thought to act as an agonist on the PGI_2 receptor of the human platelet and to desensitize specifically the PGI_2 receptor-adenylate cyclase complex (Miller & Gorman, 1979). (3) The xanthone derivative AH 6809 specifically antagonizes the inhibitory action of PGD_2 on human platelets (Keery & Lumley, 1985). It slightly potentiates the action of PGI_2 and its carba analogues. In our experiments the actions of EP 035 and EP 157 were also slightly potentiated. The exact mechanism of action of AH 6809 is not known. It also blocks PGE_2-mediated contractile responses of smooth muscle (Coleman *et al.*, 1985), and at high concentrations (300 μM) blocks aggregation of human platelets induced by U-46619 (Keery & Lumley, 1985). This anti-thromboxane effect was of no significance in our studies since ADP was used as the aggregating agent in the presence of indomethacin. (4) EP 035 and EP 157 displace [H]-iloprost binding from its specific binding sites on human platelet membranes.

In the systems described above the rank order of activity was always iloprost > 6a-carba PGI_2 > EP 157 > EP 035. The relative activities were also reasonably constant with 6a-carba PGI_2 being only 2–5 times more active than EP 157. An exact correlation of potencies is not necessarily to be expected since the systems vary considerably in their absolute sensitivity to prostacyclin-like action. For example, in the aggregation experiments using a plasma-free platelet suspension iloprost is highly effective at 0.2 nM and EP 157 at 10 nM. We presume that at these concentrations receptor occupancy for both compounds is very low and that the inhibitory mechanism is highly sensitive to small elevations in the cyclic AMP level. In contrast, comparison of complete concentration-effect curves for activation of adenylate cyclase and displacement of [^{3}H]-iloprost binding indicates that almost full occupancy is required for full effect and that the concentrations of the analogues need to be increased by some 1000 fold to achieve this. It is possible that EP 035 and EP 157 at the higher concentrations used (>1 μM in a protein-free system) may modify their own specific biochemical effects by a physical action related to their high lipophilicity. This may be of particular relevance when trying to assess whether an analogue can maximally activate adenylate cyclase – for example EP 035 on the human platelet homogenate (Figure 4). In one experiment where EP 035 and EP 157 concentrations were increased up to 100 μM bell-shaped curves were obtained.

The high lipophilicity of EP 035 and EP 157 may account for the slow onset and offset of their thromboxane blocking effects on the three isolated smooth muscle preparations. We have observed a similar phenomenon with thromboxane-like agonists of high lipophilicity (Jones *et al.*, 1982). We suggest that a stable biological effect requires equilibrium receptor occupancy to be established throughout the tissue. Less accessible receptors are only slowly occupied since the extracellular fluid is continuously depleted of compound through uptake into adjacent lipophilic domains. This mechanism is probably of little consequence in discrete cell fragments such as platelets.

In order to complete the investigation of EP 035 and EP 157 on human platelets a specific PGI_2 receptor antagonist is required. We know of no substantiated report concerning such a compound. Use of this type of compound would allow an estimation of the ability of EP 035 and EP 157 to specifically block thromboxane receptors on the human platelet.

At least two other systems, the NCB-neuroblastoma cell line and human lung tissue, repond to EP 035 and EP 157 with an elevation of their cyclic AMP content. The iloprost binding experiments suggest that this effect is due to an agonist action at the PGI_2 receptor. In the case of the human lung it would be of interest to determine which cell type(s) respond to iloprost and the EP analogues and to investigate the pharmacological actions of these agents in an intact preparation.

The discovery of EP 035 and EP 157 opens up an interesting concept of drugs with both thromboxane-blocking and PGI_2-like activity. There has been considerable interest in the development of thromboxane synthetase inhibitors since the demonstration that the vessel wall can synthesize PGI_2 from prostaglandin endoperoxides released from platelets (steal hypothesis: Moncada & Vane, 1979). When platelets are pretreated with thromboxane synthetase inhibitors such as imidazole (Moncada *et al.*, 1977) or 9,11-imioepoxyprosta-5,13-dienoic acid (Fitzpatrick *et al.*, 1979) endoperoxides are made available to the vessel wall. Thromboxane synthetase inhibitors are thought to be potentially useful anti-thrombotic agents since they not only protect against the noxious effects of TXA_2, but may result in the redirection of metabolism of prostaglandin endoperoxides to PGI_2. However, *in*

vivo, the vessel wall could be damaged or obstructed by an atheromatous plaque so that the PGI_2 synthetase enzyme cannot utilize the platelet endoperoxides. Under these conditions, EP 035 and EP 157 might prove more useful since in addition to specifically antagonizing TXA_2 they would have direct PGI_2-like anti-aggregatory activity. Furthermore, the specific thromboxane receptor antagonism produced by EP 035 and EP 157 would eliminate any vasoconstriction or residual platelet aggregation induced by the prostaglandin endoperoxides.

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