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EP 171: a high affinity thromboxane A_2-mimetic, the actions of which are slowly reversed by receptor blockade

R.L. Jones, N.H. Wilson & Ruth A. Lawrence

Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh, EH8 9JZ

1 Replacement of the four-carbon ω-terminus in 9,11-endoxy-10a-homo prostaglandin H_2 with a *p*-fluorophenoxy group produces a compound (EP 171) with very high agonist potency at TP-receptors.

2 On six isolated smooth muscle preparations EP 171 was 33–167 times more potent as a TP-receptor agonist than U-46619 (11,9-epoxymethano PGH_2); EC_{50} values ranged from 45 to 138 pM. The actions of EP 171 were difficult to study because of their slow onset and offset. For example, on the guinea-pig trachea the time required for 50% reversal of EP 171-induced contractions during washout was about 3 h.

3 On the pig pulmonary artery, a more rapidly responding preparation, it was possible to show that the TP-receptor antagonist EP 092 blocked the contractile actions of EP 171 and U-46619 to similar extents: $pA_2 = 8.09$ and 8.15 respectively.

4 EP 171 was also a very potent activator of human blood platelets, being about 90 times more potent than U-46619. Both shape change (0.1 nM) and aggregation (1 nM) were slow in onset, a profile not previously observed for a thromboxane A_2-mimetic.

5 When potencies at TP-, EP_1-(guinea-pig fundus) and FP-(dog iris sphincter) receptors were compared, EP 171 showed a higher specificity as a TP-receptor agonist than either STA_2 or U-46619. These studies also showed that contrary to earlier reports, the guinea-pig fundus does contain TP-receptors mediating muscle contraction. However, the maximal response due to activation of TP-receptors was only about 35% of the PGE_2 maximum.

6 Established responses to EP 171 were slowly reversed following addition of a high concentration of a TP-receptor antagonist (EP 092, GR 32191 or BM 13177). Faster reversals of three less potent 16-*p*-halophenoxy prostanoids and U-46619 were obtained. Half-times for offset (and onset) of agonist action appeared to correlate with potency rather than with lipophilicity.

7 Competition between the agonists and a radio iodinated PTA_2 derivative ([^{125}I]-PTA-OH) for binding to TP-receptors on intact human platelets was studied. IC_{50} values correlated well with aggregating potency, EP 171 having the lowest IC_{50} of 2.9 nM. The true K_i for EP 171 may be about 1 nM if both its racemic nature and reduction of initial free ligand concentration due to TP-receptor binding are taken into account.

8 It is concluded from a comparison of agonist potency rankings that subclassification of the TP-receptor is not warranted at this time. The factors that may be responsible for the slow kinetics of EP 171 action are discussed.

Introduction

Manipulation of the chemical structure of a transmitter substance or hormone can result in substantial loss or, less frequently, enhancement of potency. While clearly the maximum degree of loss of potency is absolute, the maximum degree of enhancement has yet to be defined. Our early studies of TP-receptor agonists (thromboxane A_2 mimetics; see Kennedy *et al.*, 1982; 1983, for prostanoid receptor nomenclature) showed that alteration of the ω-chain structure of compounds with weak agonist activity could dramatically increase potency. For example, it was observed that the replacement of the terminal four-carbon unit of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) with a *p*-fluorophenoxy group produced an analogue

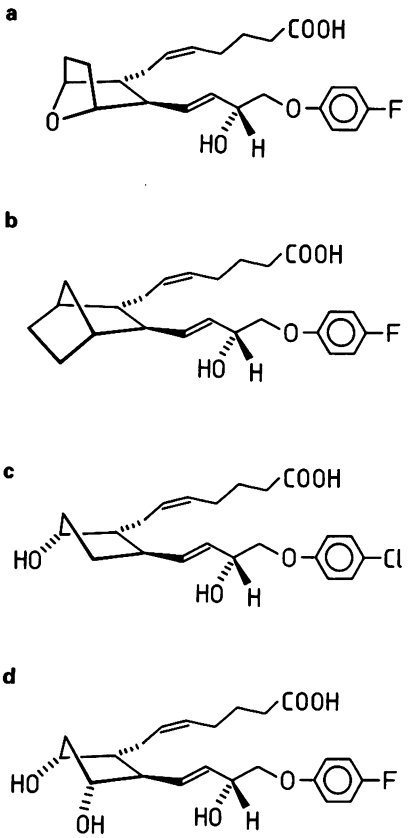

Figure 1 Structures of the 16-*p*-halophenoxy-ω-tetranor prostanoids examined in this study: (a) EP 171 (*rac*), (b) EP 031 (*rac*), (c) 16-*p*-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ (*rac*) and (d) 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ (*nat*).

(structure d in Figure 1) with 50 fold greater contractile potency on the isolated aorta of rabbit and saphenous vein of dog (EC_{50} = 30 nM) (Jones & Marr, 1977). When a similar substitution was made on all-carbon ring analogues of the prostaglandin endoperoxide PGH_2, partial agonist activity was converted into potent full agonism. For example, EP 031 (Figure 1) has an EC_{50} value for contraction of rabbit aorta, dog saphenous vein and guinea-pig trachea of about 1 nM (Jones *et al.*, 1982). We were curious to know therefore whether the 16-*p*-fluorophenoxy-ω-tetranor modification would greatly enhance the potency of a prostanoid which was already a potent full agonist of TP-receptors (EC_{50} < 10 nM).

Our choice of molecule was influenced by the remarkably slow onset and offset of EP 031 action on isolated smooth muscle preparations. A contact time of 2–3 h is often required to achieve a stable submaximal response and the return to resting tension even with continuous wash-out of the organ bath takes 4–5 h (Jones *et al.*, 1982). We assumed that the slow onset is mainly due to removal of the highly lipophilic analogue from the extracellular space into adjacent lipid domains. Thus diffusion of the agonist to membrane receptors in the centre of the preparation is retarded and equilibrium occupation of the receptor pool is only slowly attained. Offset of action is correspondingly slow because the loss of agonist from the lipid reservoir maintains the extracellular space concentration. EP 031 is also a potent activator of human platelets (Wilson *et al.*, 1982), but its rate of action (shape change or aggregation) is only marginally slower than other TP-receptor agonists. This is not surprising if we suppose that agonist molecules, irrespective of their lipophilicity, have ready access to surface membrane receptors of discrete cell fragments in a well-stirred system. We felt therefore that a polar bicyclic ring system would favour a rapid onset/rapid offset action on smooth muscle preparations by increasing the overall water solubility of the molecule. By this reasoning, CTA_2(9,11-carba-11a-carba TXA_2), the all-carbon ring analogue of TXA_2 (Lefer *et al.*, 1980), was deemed to be an unsuitable candidate for the parent molecule. 9,11-Endoxy-10a-homo PGH_2 was much more attractive since our chemical work on precursor molecules showed that the 7-oxabicyclo[2.2.1]heptane ring endowed considerable water solubility and a preliminary report in the literature (Sprague *et al.*, 1983) indicated high agonist potency on guinea-pig trachea and human platelets. The 16-*p*-fluorophenoxy-ω-tetranor derivative of 9,11-endoxy-10a-homo PGH_2 (Figure 1) is coded EP 171 and this paper describes our attempts to determine its potency, specificity and kinetics on isolated smooth muscle preparations and human platelets.

Methods

Isolated smooth muscle preparations

Thoracic aortae were removed from male rats (250–300 g) killed by stunning and exsanguination and from male rabbits (2–3 kg) killed by exsanguination under pentobarbitone anaesthesia. Segments of saphenous vein were obtained from dogs under pentobarbitone anaesthesia, and eyes were removed from the same animals killed by air embolism. Lobar pulmonary arteries were dissected from lungs of pigs (30–35 kg) killed by exsanguination under pentobar-

bitone anaesthesia. Trachea and stomach were removed from guinea-pigs (400–700 g) of either sex killed by stunning and exsanguination. Bullock eyes were obtained from the abbatoir.

Rings, 3 mm wide, of trachea and blood vessels were suspended between stainless steel hooks in 10 ml organ baths and tension changes were recorded with Grass FT03 force displacement transducers linked to a Grass Polygraph. Tension changes in the sphincter pupillae of the iris and strips of fundic stomach (ventral surface) were measured with the same recording system, connections being made with fine silk thread. The Krebs bathing solution contained (mmol l^{-1}): NaCl 118, KCl 5.4, $MgSO_4$ 1.0, $CaCl_2$ 2.5, NaH_2PO_4 1.1, $NaHCO_3$ 25 and dextrose 10, and was gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. In addition, for the pig pulmonary artery and the dog and bullock iris preparations, indomethacin (1 μM) was present and for the guinea-pig trachea and fundus indomethacin (1 μM) and atropine (20 nM).

As a general procedure, a 1 h equilibration procedure was followed by the cumulative addition of doses of the standard agonist. After washout of the organ bath by upward displacement the standard agonist cumulative dose sequence was repeated. With fast onset/fast offset test compounds a cumulative dose sequence was performed and then a final sequence of standard agonist doses was applied. With EP 171 and EP 031 the organ bath system was perfused with a suitable concentration of the agonist and when a stable level of contraction had been reached the flow was stopped. A high concentration of 11,9-epoxymethano PGH_2 (U-46619, 1 μM) was then added to establish the tissue maximum response. This procedure is termed the 'single + maximum dose method'.

Platelet activation

The preparation of human platelets for shape change/aggregation measurements has been described previously by us (Armstrong *et al.*, 1985). In this single wash procedure the platelet pellet from the centrifugation of platelet-rich plasma was suspended in Ca^{2+}-free Krebs solution (composition as above) and maintained at 37°C. The plasma protein concentration was about 1 mg ml^{-1} (from u.v. absorbance at 280 nm). Additional washing steps designed to remove the remaining plasma protein tend to reduce the sensitivity of the platelets to aggregating agents.

Shape change and aggregation were measured with a modified Cary 118C spectrophotometer (incident light wavelength = 600 nm). Each cuvette, containing 1.0 ml platelet suspension, 1.0 ml Krebs solution and 0.4 ml 0.9% NaCl solution, was held in a heated jacket at 37°C and stirring was achieved with a stainless steel rod revolving at 1000 r.p.m. Aggregating agents were added in 50 or 100 μl of 0.9% NaCl solution.

Ligand binding

Inhibition of the binding of radio iodinated 13-aza-13,14-dihydro-16-(p-hydroxy-m-iodophenyl)-ω-tetranor PTA_2 ([^{125}I]-PTA-OH) to washed human platelets was measured essentially according to the method of Narumiya *et al.* (1986). The platelet pellet obtained as described above was suspended in assay buffer (100 mM NaCl, 5 mM dextrose, 1 μM indomethacin and 50 mM Tris-HCl, pH 7.4) and PGI_2 addition, centrifugation and resuspension of the pellet was repeated. The final incubation mixture (200 μl in 1.5 ml Eppendorf tubes) contained about 5×10^7 platelets, 0.1 nM [^{125}I]-PTA-OH (75 TBq $mmol^{-1}$, Amersham), 4 nM cicaprost (Stürzebecher *et al.*, 1986) and a variable concentration of the displacing agent. After incubation for 30 min at 37°C, the tubes were centrifuged at 16,000 *g* for 1 min and then transferred onto ice. The supernatant was rapidly removed by tapping onto absorbant paper and the pellet was washed with 1 ml of ice-cold assay buffer. Radioactivity in each pellet was measured with a LKB Universal Gamma Counter.

Each treatment was run in triplicate and non-specific binding was determined with 1 μM ONO 11120 (13-aza-13,14-dihydro-16-phenyl-ω-tetranor PTA_2), a close analogue of the radioligand (Katsura *et al.*, 1983). Inhibition curves for each prostanoid were obtained on platelets from four separate donors. IC_{50} values correspond to K_i values since the radioligand concentration (0.1 nM) is much less than the K_d of the radioligand (20 nM) (Narumiya *et al.*, 1986).

Partition coefficients and h.p.l.c. retention volumes

Compounds were partitioned between 5 ml of 0.1 M $NaHPO_4/K_2HPO_4$ buffer pH 7.4 and 5 ml of chloroform. Brief centrifugation was performed to reduce the contamination of each phase through emulsification. The concentration in each phase was determined by u.v. spectroscopy or gas chromatography after suitable derivatisation. In the case of EP 031 and EP 092 the concentration remaining in the aqueous phase was insufficient for accurate measurement. The partition was therefore conducted between 0.1 M $NaHCO_3/Na_2CO_3$ buffer pH 9.0 and chloroform and the partition coefficient corrected to

pH 7.4, by use of the Henderson-Hasselbach equation and assuming a pKa of 5.0 for each compound.

As a corroborative measure, retention volumes for the chromophore-containing compounds was determined on a reversed-phase h.p.l.c. system. The octadecylsilane-bonded column (Partisil PXS 10/25 ODS, Whatman) was equilibrated with methanol/water/acetic acid 60:40:0.1 (by vol.) at a flow rate of 1 ml min^{-1}. Retention volumes (expressed as % of column volume) were BM 13177 (4-[2'-benzenesulphonamido ethyl)-phenoxy acetic acid) 97, 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ 128, 16-*p*-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ 188, EP 171 189, EP 092 488 and EP 031 517.

Compounds

The following compounds were prepared in our laboratory: (*rac*)9α,11α-epoxy-10a-homo-15S-hydroxy-prosta-5Z,13E-dienoic acid (9,11-endoxy-10a-homo PGH_2) and its 16-*p*-fluorophenoxy-ω-tetranor derivative (EP 171), (*rac*)9α,11α-ethano-15S-hydroxy-16-*p*-fluorophenoxy-ω-tetranor-prosta-5Z,13E-dienoic acid (EP 031), (*rac*)9α,11α-ethano-1-methyl-13(N-phenylthio-carbamoyl)hydrazono-ω-heptanor-prosta-5Z-enoic acid (EP 092) and (*nat*)16-*p*-fluorophenoxy ω-tetranor $PGF_{2\alpha}$.

STA_2 (11a-carba-9,11-thia TXA_2) and ONO 11120 were gifts from the ONO Company, Japan. BM 13177 was a gift from Boehringer – Mannheim, W. Germany, misoprostol a gift from Searle Ltd., U.S.A. and GR 32191 (1α-(6'-carboxyhex-3'Z-enyl)-2β-(N-piperidino)-3α-hydroxy-5α-(4''-biphenylyl methoxy)-cyclopentane) a gift from Glaxo, U.K. 11,9-Epoxymethano PGH_2 (U-46619) was purchased from Upjohn Diagnostics, U.S.A.

SC 19220 (10-(acetyl hydrazino carbonyl)-8-chloro-10,11-dihydrodibenz (b,f)(1,4) oxazepine) and SC 25191 (10-(n-butyryl hydrazino carbonyl)-8-chloro-10,11-dihydrodibenz (b,f)(1,4) oxazepine) were gifts from Searle, U.S.A. They were added to the organ bath dissolved in ethanol such that the final ethanol concentration in the organ bath was 18 mM when the concentrations of the blockers were 30 and 10 μM respectively.

Results

Actions at TP-receptors in smooth muscle

The potency of EP 171 was compared with that of U-46619, the most commonly used standard agonist for TP-receptor studies, on six isolated smooth muscle preparations: rabbit aorta, rat aorta, dog saphenous vein, pig pulmonary artery, guinea-pig trachea (all ring preparations) and the bullock iris sphincter (two strips obtained from the one eye). The mean EC_{50} value for U-46619 lay between 4 and 12 nM on each preparation. EP 171 was highly potent and produced contractile effects at concentrations of 0.1 nM and less. To our surprise its rates of onset and offset of action were comparable to or even slower than those of EP 031. Indeed it was only on the most rapidly responding preparation, the pig pulmonary artery, that concentration-response relationships could be obtained by cumulative addition of EP 171 doses. The results, shown in Figure 2, indicate that EP 171 is about 100 times more potent than U-46619, about 50 times more potent than its parent 9,11-endoxy-10a-homo PGH_2 and about 20 times more potent than STA_2, a close structural analogue of TXA_2 (Katsura *et al.*, 1983).

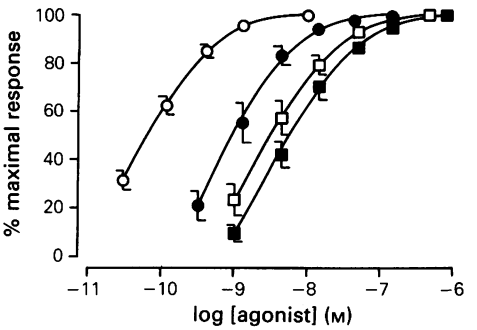

Figure 2 Log concentration-response curves for contractile action of EP 171 (○), STA_2 (●), 9,11-endoxy-10a-homo PGH_2 (□) and U-46619 (■) on the pig isolated pulmonary artery. Mean responses are derived from experiments on 4 preparations each from a different pig; vertical bars show s.e.mean.

On the other five smooth muscle preparations the onset of action of EP 171 was so slow that it was necessary to employ the single + maximum dose technique (Jones *et al.*, 1982) for the construction of concentration-response relationships. In brief, preparations which responded reproducibly to U-46619 were exposed to a single concentration of EP 171 for 100–300 min before a maximum dose of U-46619 was added. Each preparation yields a single data point and 15–20 data points are required to give an accurate log concentration-response curve for EP 171. EP 171 was a full agonist on each of the five preparations; EC_{50} values are given in Table 1. An equipotent molar ratio (e.p.m.r., U-46619 = 1.0) for EP 171 was obtained from each preparation where the EP 171 response fell between 20 and 80% of the

Table 1 Agonist potencies of EP 171, STA_2 9,11-endoxy-10a-homo PGH_2 and U-46619 at TP-receptors in smooth muscle and human platelets

Preparation	*EC_{50} for EP 171* (pM)	*Equipotent molar ratio* (± s.e.mean, U-46619 = 1.0) *EP 171*	*STA_2*	*9,11-Endoxy-10a-homo PGH_2*
Rabbit aorta	138	0.0136 ± 0.0025 (8)*	0.23 ± 0.06 (4)	0.62 ± 0.08 (4)
Rat aorta	45	0.0094 ± 0.0006 (7)*	0.15 ± 0.02 (4)	0.63 ± 0.07 (4)
Pig pulmonary artery	70	0.0093 ± 0.0008 (4)	0.21 ± 0.03 (4)	0.50 ± 0.06 (4)
Dog saphenous vein	120	0.0302 ± 0.0034 (11)*	0.30 ± 0.05 (4)	0.69 ± 0.05 (4)
Guinea-pig trachea	57	0.0096 ± 0.0012 (9)*	0.35 ± 0.04 (4)	0.44 ± 0.04 (4)
Bullock iris sphincter	72	0.0060 ± 0.0005 (5)*	0.080 ± 0.002 (4)	0.38 ± 0.02 (4)
Human platelets shape change	51	0.0110 ± 0.0020 (6)	—	—
aggregation	—	—	0.30 ± 0.05 (4)†	0.96 ± 0.05 (4)†

Values in parentheses refer to the number of preparations used.
* Single + maximum dose method was employed.
† Data from Jones *et al.*, 1987.

maximum response and a comparison could therefore be made with the corresponding U-46619 log concentration-response curve (Table 1). EP 171 is 33–167 times more potent than U-46619, 23–67 times more potent than 9,11-endoxy-10a-homo PGH_2 and 10–40 times more potent than STA_2.

Submaximal (<80%) responses to EP 171 on all six preparations were completely inhibited by the TP-receptor antagonists EP 092 (1 μM) (Armstrong *et al.*, 1985) and BM 13177 (30 μM) (Patscheke & Stegmeier, 1984). It is obviously difficult when using EP 171 as agonist to obtain accurate pA_2 values for TP-receptor antagonists by the Schild procedure (Arunlakshana & Schild, 1959). However, in the case of the pig pulmonary artery an 80% maximum response to EP 171 will return to less than 5% of maximum after 4 h continuous displacement of the bathing fluid and this allows a second cumulative series of doses to be applied. It was therefore possible to obtain a dose-ratio for antagonism of EP 171 action on one preparation and a corresponding measure of the change in sensitivity to EP 171 with time on a second (control) preparation. Using preparations from four separate pigs, the mean dose ratio for 0.25 μM EP 092 versus EP 171 was 32.0 ± 4.6 (s.e.mean) and the corresponding control mean dose-ratio 1.12 ± 0.08. The dose-ratio for 0.25 μM EP 092 versus U-46619 determined on parallel preparations from the four pigs was 36.2 ± 6.1 and the control value 1.00 ± 0.07. Using the Schild equation, a pA_2 value of 8.09 was obtained for the EP 092/EP 171 interaction and 8.15 for the EP 092/U-46619 interaction. These values (although obtained with a single antagonist concentration) are close to previous values obtained for the EP 092/U-46619 interactions at TP receptors on dog saphenous vein (7.94) and guinea-pig trachea (7.96) (Armstrong *et al.*, 1985).

Actions at FP- and EP_1-receptors in smooth muscle

The dog iris sphincter contracts to low concentrations of $PGF_{2\alpha}$ (mean EC_{50} in these experiments = 3 nM) and appears to contain only FP-receptors (Dong & Jones, 1982; Kennedy *et al.*, 1983). Responses to $PGF_{2\alpha}$ were rapid in onset and offset but tended to fade if greater than 75% of the maximum and the contact time was prolonged. EP 171, STA_2 and U-46619 behaved as full agonists. The e.p.m.rs ($PGF_{2\alpha}$ = 1.0) are given in Table 2. The action of EP 171 was slightly slower in onset and offset than $PGF_{2\alpha}$ but similar to one of the most potent FP-receptor agonists (*rac*)16-*m*-trifluoromethylphenoxy-ω-tetranor $PGF_{2\alpha}$ (ICI 81008) (Table 2). EP 092 (3 μM) had little blocking action on either $PGF_{2\alpha}$ or EP 171 (dose ratio = 1.3–1.6, $n = 3$ in each case).

Although the guinea-pig trachea and the bullock iris sphincter contain EP_1-receptors mediating muscle contraction, estimation of the EP_1-receptor agonist potency of EP 171 on these preparations would require a very effective (and possibly irreversible) blockade of TP-receptors. We therefore decided to examine the activity of EP 171 on the guinea-pig stomach fundus, a EP_1-receptor preparation thought to be devoid of TP-receptors (Kennedy *et al.*, 1983).

Table 2 Potency and specificity of prostanoid agonists

Agonist	*Equipotent molar ratio* (± s.e.mean) *Dog iris* ($PGF_{2\alpha} = 1.0$)	*Guinea-pig fundus*† ($PGE_2 = 1.0$)	*Ratio of EC_{50} values** *Dog iris/rat aorta*	*Guinea-pig fundus*†*/rat aorta*
EP 171	44 ± 4 (6)	268 ± 32 (4)	3100	14000
STA_2	151 ± 30 (4)	243 ± 53 (4)	630	1700
U-46619	196 ± 12 (4)	3230 ± 520 (4)	130	1500
ICI 81008	0.15 ± 0.05 (4)	>1000 (4)	0.00015	—
16,16-dimethyl PGE_2	>100 (4)	0.115 ± 0.015 (4)	—	0.0035

Values in parentheses are numbers of observations.
* A high ratio indicates specificity for TP-receptors over FP-receptors (dog iris) or EP_1-receptors (guinea-pig fundus).
† 3 μM EP 092 present.

The fundus was highly sensitive to the standard agonist PGE_2. On 12 preparations the shape of the PGE_2 log concentration-response curve corresponded to a single sigmoid (EC_{50} = 1.2–5.2 nM). EP 171 however showed a biphasic log concentration-response curve (Figure 3a). The more potent contractile component covered the range 0.1–10 nM and had a maximum of about 35% of the PGE_2 maximum (all % maximum values relate to the PGE_2 maximum). The EP 171 responses were slow in onset and offset and to ensure that a build up of metabolites in the organ bath did not augment the responses, the EP 171 solution was continuously replaced using a roller pump. Above 10 nM, EP 171 added as discrete doses elicited contractile responses which quickly reached a stable level (~5 min): on wash-out of the organ bath these responses decayed rapidly, but only to the 30–40% maximum response level. It seemed likely that EP 171 was specifically activating TP-receptors at concentrations of 10 nM and below, whilst at higher concentrations both TP- and EP_1-receptor activation contributed to the contractile response. The following observations support this hypothesis.

The EP_1-receptor antagonist SC 25191 (10 μM) (Sanner *et al.*, 1973) did not affect the more potent component of EP 171 action but reduced the less potent component (Figure 3a). The rightward shift of the EP 171 curve at the 50% maximal response level is similar to the shift of the PGE_2 curve due to the presence of 10 μM SC 25191: PGE_2 dose-ratio = 13.6 ± 1.4 (n = 5). In contrast, EP 092 (3 μM) did not affect responses to PGE_2 (dose ratio = 1.0–1.5, n = 4), but abolished responses to concentrations of EP 171 up to 10 nM (Figure 3a, b). EP 092 also abolished responses to STA_2 and U-46619 provided these were less than 30% of the PGE_2 maximum (Figure 3a, b). Within the 5–25% maximum range, EP 171 was about 30 and 70 times more potent than STA_2 and U-46619 respectively; these values are similar to relative potencies found on the other preparations containing TP-receptors (Table 1). In the experiments of Kennedy *et al.* (1983) a 300 μM concentration of SC 19220, a close analogue of SC25191, abolished responses to U-46619, leading to the suggestion that the agonist action was entirely due to activation of EP_1-receptors. We feel that SC 19220 may not be specific at this high concentration; in our hands 30 μM SC 19220 behaved similarly to 10 μM SC 25191, blocking PGE_2 responses but not responses to EP 171 or U-46619 when these were less than 30% of the PGE_2 maximum.

Equipotent molar ratios (Table 2) were calculated from log concentration-response curves obtained in the presence of 3 μM EP 092 (Figure 3b). We thought it possible however that the higher concentrations of the three potent TP-receptor agonists might overcome the EP 092 block. As a corroborative measure, therefore, we also obtained relative potencies on EP_1-receptors during continuous near-maximal activation of the thromboxane-sensitive system. For this purpose, the preparations were continuously bathed with 10 nM EP 171 and concentration-response relationships to PGE_2 and the three thromboxane A_2-mimetics determined. Figure 4 shows a typical experiment and Figure 3c the combined data. E.p.m.rs (PGE_2 = 1.0) were calculated at the 70% maximal response level: EP 171 170 ± 22, STA_2 131 ± 8, U-46619 2350 ± 380 (n = 4); the EP 092 blockade would appear to have been adequate.

Activation of human platelets

EP 171 is the most active thromboxane A_2 mimetic with respect to the aggregation of human platelets *in*

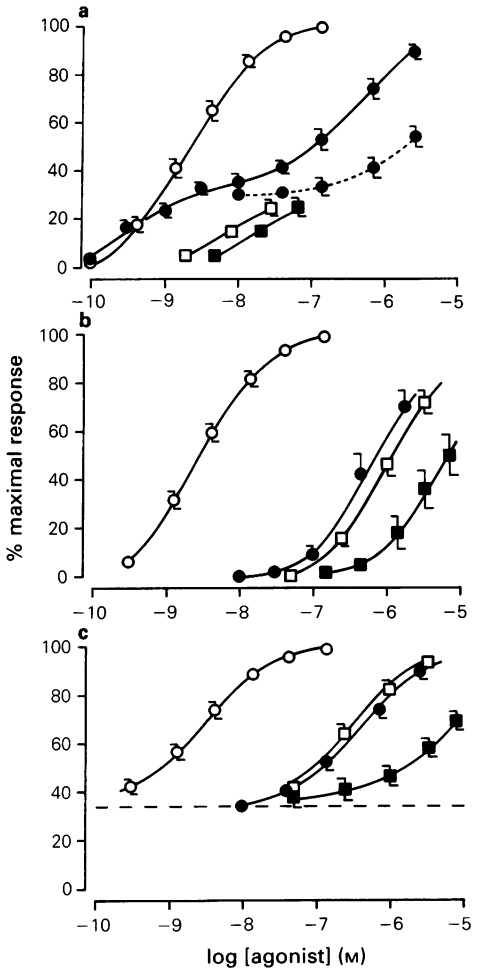

Figure 3 Log concentration-response relationships on the guinea-pig stomach fundus. Mean responses for PGE_2 (○) ($n = 8$–12), EP 171 (●), STA_2 (□) and U-46619 (■) ($n = 4$ for each) are shown; vertical bars show s.e.mean. In the group of experiments depicted in (a) the agonists act alone except for EP 171 in the presence of 10 μM SC 25191 (- - - -). In (b) the TP-receptor antagonist EP 092 (3 μM) was present and in (c) 10 nM EP 171 was present throughout, producing near-maximal activation of the thromboxane-sensitive system.

vitro that we have encountered so far. However, responses to EP 171 were quite unlike those of any other thromboxane A_2 mimetic in that primary reversible aggregation waves were never produced. With low concentrations (0.5–1 nM) the onset of aggregation was delayed such that the full extent of the shape change response was always seen (Figure 5a). Slowly developing aggregation induced by EP 171 showed little tendency to reverse. Increasing the EP 171 concentration led to increasingly faster onset of agrregation such that at 50 nM the EP 171 profile was very similar to that of a maximal irreversible response to U-46619. It was not possible to make accurate potency comparisons when the time courses of the responses to the two agonists were so different.

Shape change responses to EP 171 were also slow in onset compared to those of U-46619 (Figure 5b). However, EP 171 did produce graded stable submaximal responses and this allowed comparison with the standard agonist. EP 171 had an EC_{50} value of about 50 pM and an e.p.m.r. of 0.011 (U-46619 = 1.0) (Table 1). The cyclo-oxygenase inhibitor indomethacin (1 μM) did not influence the profile of activity of EP 171.

Reversal of agonist action by TP-receptor blockade

The observation that the smooth muscle contractile action of EP 171 mediated by TP-receptors was slowly reversed by washing whereas its contractile actions through FP- and EP_1-receptors were quickly reversed suggested to us that the interaction with the TP-receptor, rather than sequestration into lipid, could be the dominant factor in the rate of action of EP 171. We supposed that an agonist with activity at concentrations below 100 pM must have a reasonably low equilibrium dissociation constant (K_d) and, by conventional wisdom, the avid binding would be reflected in a low dissociation rate constant (k_2). It was therefore of interest to determine how quickly a high concentration of a TP-receptor antagonist would reverse an established submaximal response to EP 171 in comparison with less potent TP-receptor agonists.

Measurements of reversal rates on smooth muscle were made on the rabbit aorta and guinea-pig trachea. Each preparation was exposed to a single concentration of agonist sufficient to produce a 50–70% maximum response and the time (t_{on}) for the response to reach 50% of its final level was measured. The preparation was then treated in one of three ways: (a) washed by continuous upward displacement of the bathing fluid, (b) exposed to a TP-receptor antagonist (EP 092 or BM 13177) and (c) exposed to a physiological antagonist (the PGE analogue misoprostol on the guinea-pig trachea and atrial natriuretic peptide, ANP, on the rabbit aorta). The time (t_{off}) for the response to decay to 50% of its original magnitude was measured in each case.

In addition to EP 171, U-46619 and three other 16-*p*-halophenoxy prostanoid agonists, 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$, 16-*p*-chloro-

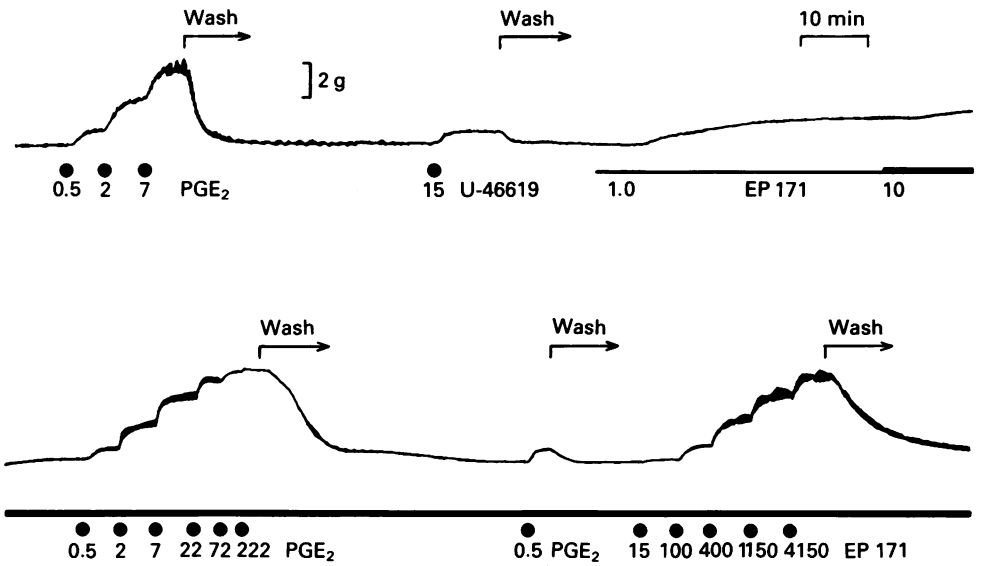

Figure 4 Estimation of the agonist potency of EP 171 at EP_1-receptors in the guinea-pig fundus strip: 1 μM indomethacin and 20 nM atropine were present throughout. EP 171 was present in the bathing fluid as indicated by the solid bars. Cumulative concentrations (nM) of PGE_2 and EP 171 are shown. The single dose of PGE_2 was added to ensure that the preparation had not been desensitized by the preceding high doses of PGE_2.

phenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ and EP 031 (Figure 1) were investigated. On the guinea-pig trachea contractile responses to the two PGF analogues are due to activation of both TP- and EP_1-receptors (Jones *et al.*, 1982) and consequently half-times for these two agonists were measured in the presence of 30 μM SC 19220 (see Dong *et al.*, 1986); this treatment did not affect the potencies or rate profiles of EP 031 and EP 171.

The results are shown in Figure 6a, b. The five agonists have been arranged from left to right in order of increasing potency. For example, the concentrations used to produce matching submaximal responses on the rabbit aorta were typically 15 nM for 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$, 7.5 nM for U-46619, 2.5 nM for 16-p-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$, 0.6 nM for EP 031 and 0.1 nM for EP 171. There is a reasonably good correlation between agonist potency and both t_{on} and wash-t_{off}. The correlation between lipophilicity and t_{on} and wash-t_{off} is poorer; partition coefficients (PC) between chloroform and pH 7.4 water are given in Table 3. Measurements were also made on a diastereoisomer of EP 171, in which the ω-chain is *cis* to the α-chain and the 15-hydroxyl is β-orientated. This compound is slightly more lipophilic than EP 171 but about 500 times less potent (e.p.m.r. = 4.8 $\pm$ 1.3, $n = 4$; U-46619 = 1.0, guinea-pig trachea). It showed rapid onset/offset characteristics similar to 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$.

The slowest decay of contractile action following addition of the TP-receptor antagonists is seen with EP 171 on both preparations. The similar half-times for the three least potent agonists may reflect the rate at which the antagonist penetrates the tissue and binds to TP-receptors and/or the rate at which the contractile process is reversed once the stimulatory input has been removed. It should be noted that the two antagonists differ in affinity and particularly in lipid solubility. EP 092 has a reasonably high affinity ($pA_2 = 7.26$ and 7.96 for rabbit aorta and guinea-pig trachea respectively) and is highly lipophilic (PC = 1900), whereas BM 13177 is a weaker blocker ($pA_2 = 6.24$ and 6.30 respectively, our results using U-46619 as agonist) and has low lipid solubility (PC = 0.013). Contractions to each of the five agonists were rapidly reversed by the physiological antagonists.

Similar kinetic studies were performed on washed human platelets using shape change (70–80% of maximum) as the response parameter (Figure 6c). However, wash-t_{off} could not be determined since there is no satisfactory method for rapidly removing the agonist from the solution bathing the platelets whilst simultaneously recording light transmission.

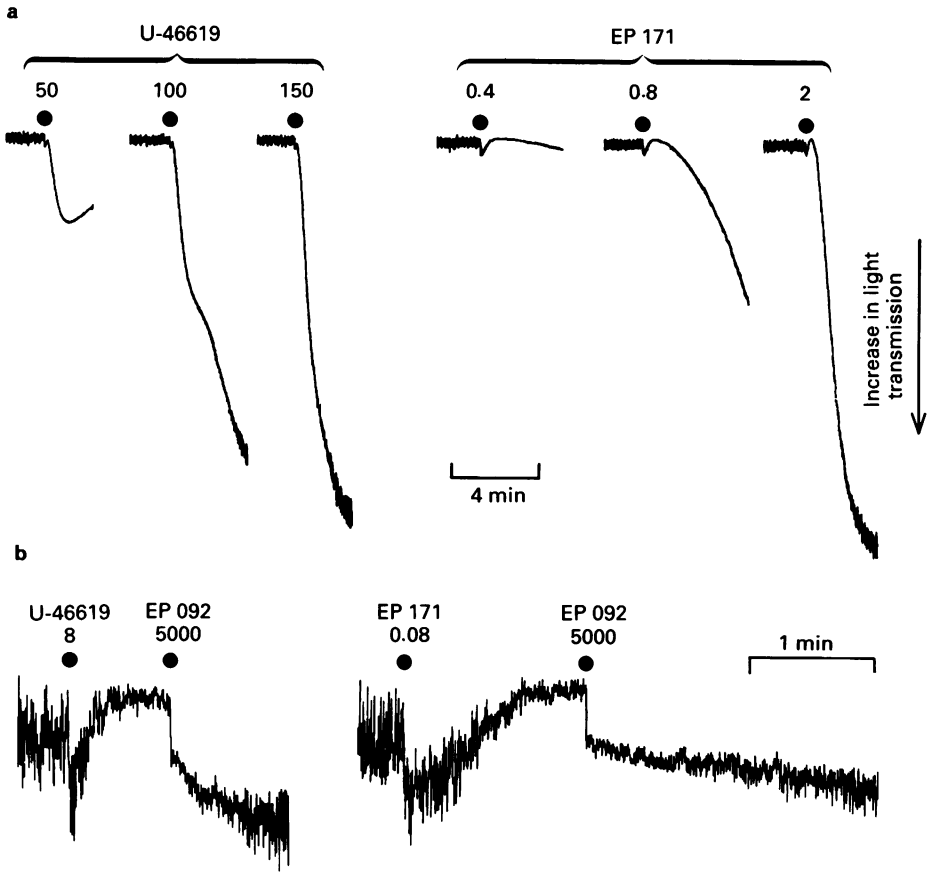

Figure 5 Comparison of the stimulant action of EP 171 and U-46619 on washed human platelets. Light transmission tracings from two experiments. Cuvette concentrations (nM) are indicated. (a) The rapid change in signal strength on addition of drug is due to dilution of the cuvette contents, the upward deflection with loss of oscillations to the platelet shape change and the downward deflection to aggregation. (b) Shape-change responses recorded at higher gain and faster chart speed than in (a). The TP-receptor antagonist EP 092 rapidly reverses the shape change induced by U-46619, but only slowly reverses the EP 171 response.

Reversal by a third TP-receptor antagonist, GR 32191 (Lumley *et al.*, 1987), was also studied. At a concentration of 30 μM, the BM 13177 blockade is surmountable and the U-46619 dose ratio is about 50 ($pA_2 = 6.2$). With 5 μM EP 092 ($pA_2 = 7.9$) and 5 μM GR 32191 ($pA_2 = 8.8$) the U-46619 dose ratio is greater than 300. The five agonists have the same potency ranking on the platelet system as on the smooth muscle preparations: EC_{50} values are given in Table 3. Responses to the three weakest agonists were rapid in onset and also in offset with all three receptor antagonists and with the physiological antagonist PGE_1 (50 nM) (Figure 6c). Responses to EP 031 were slightly slower in onset and offset (except with PGE_1). EP 171 showed the slowest onset and the slowest offset due to receptor blockade (Figure 5b); its rate of reversal with PGE_1 did not differ from that of the other four agonists.

Inhibition of [^{125}I]-PTA-OH binding to washed human platelets

The binding constants (K_i) for the interaction of U-46619 and the four *p*-halophenoxy prostanoids with the human platelet TP-receptor were estimated from concentration-inhibition curves using [^{125}I]-PTA-OH as radioligand (Figure 7 and Table 3). The ranking of K_i values correlates well with the potency of the analogues as inducers of the platelet shape change.

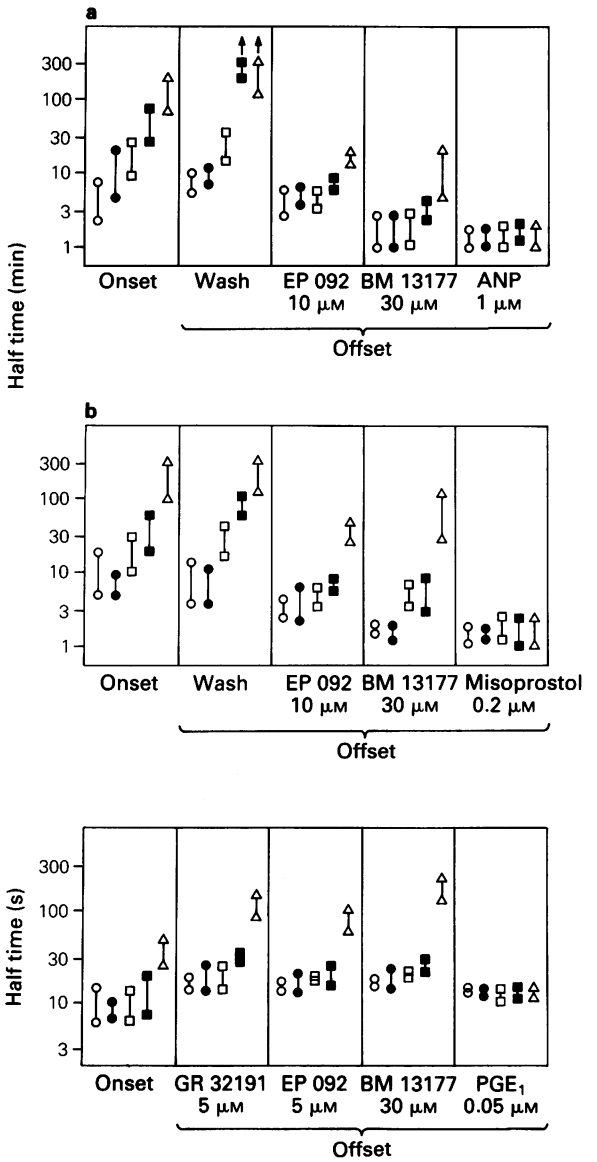

Figure 6 Half times for onset and offset of action at TP-receptors in (a) rabbit aorta, (b) guinea-pig trachea and (c) human platelets (shape change). The agonists are 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ (○), U-46619 (●), 16-*p*-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ (□), EP 031 (■) and EP 171 (△). The ranges for 12–16 separate observations for onset and 4 observations for each offset regime are shown. The arrows in the EP 031 and EP 171 columns for offset due to washing on the rabbit aorta indicate that 2 of the 4 preparations had not relaxed to 50% of the original response level after 300 min of continuous washing. Note the different ordinate scale for the platelet observations.

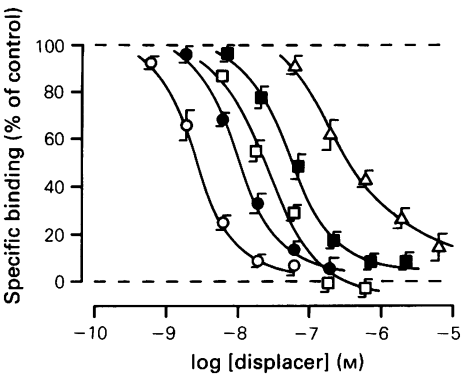

Figure 7 Inhibition of [^{125}I]-PTA-OH binding to intact human platelets by EP 171 (○), EP 031 (●), 16-*p*-chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$ (□), U-46619 (■) and 16-*p*-fluorophenoxy-ω-tetranor $PGF_{2\alpha}$ (△). Means ($n = 4$) are shown; vertical bars show s.e.mean.

Discussion

Comparison of EP 171 with its natural ω-chain parent convincingly demonstrates the ability of the 16-*p*-fluorophenoxy substituent to enhance agonist potency at TP-receptors, even when the parent compound itself is a potent full agonist. Indeed EP 171 is the most potent thromboxane A_2 mimetic reported so far and its EC_{50} values of 45–138 pM compare favourably with those of the most potent agonists on other receptor systems.

When agonist activity at TP-receptors is compared with that at FP- or EP_1-receptors, EP 171 has a higher specificity as a TP-receptor agonist than either U-46619 or STA_2. This is shown in Table 2, where the ratio of EC_{50} values on a TP-receptor preparation (rat aorta) and either a FP-receptor (dog iris) or a EP_1-receptor (guinea-pig fundus) preparation has been calculated for each agonist. The larger the ratio the greater is the specificity as a TP-receptor agonist. For the purpose of distinguishing between TP- and FP-receptors the agonist combination of EP 171 and ICI 81008 could be very useful. In the case of TP-receptors and EP_1-receptors EP 171 in combination with 16,16-dimethyl PGE_2 would suffice. Although we suspect that the development of TP-receptor agonists with greater potency than EP 171 will be difficult, increasing the specificity of action may be possible by a reduction in potency at other prostanoid receptors. EP 171 does after all activate FP- and EP_1-receptors at concentrations of 50–150 nM and could hardly be classed as a low potency agonist.

The relative potencies (and to a large extent the absolute potencies) of EP 171, STA_2, 9,11-endoxy-

Table 3 Comparison of U-46619 and *p*-halophenoxy prostanoids in terms of platelet activation, inhibition of [^{125}I]-PTA-OH binding and partition coefficient

	Human platelets		
Agonist	*EC_{50} for shape change* (nM)	*Inhibition of [^{125}I]-PTA-OH binding: K_i* (nM)	*Partition coefficient: $CHCl_3/H_2O$, pH 7.4*
EP 171	0.065 ± 0.011	2.9 ± 0.4	10
EP 031	0.55 ± 0.08	11.0 ± 1.0	1150
16-*p*-Chlorophenoxy-ω-tetranor-11-deoxy $PGF_{2\alpha}$	2.7 ± 0.6	23 ± 4	3.0
U-46619	5.4 ± 0.9	69 ± 14	15
16-*p*-Fluorophenoxy ω-tetranor $PGF_{2\alpha}$	27 ± 8	440 ± 32	0.029

Values on human platelets are means ± s.e.mean of 4 determinations.

10a-homo PGH_2 and U-46619 as TP-receptor agonists on the eight preparations studied here are quite similar (guinea-pig fundus is included). When these results are combined with our earlier structure-activity data (Jones *et al.*, 1982; Armstrong *et al.*, 1985) there appears to be no obvious division of the TP-receptor into subtypes on the basis of different agonist rankings.

The slow onset and offset of EP 171 action on TP-receptor preparations rules out any possibility of this compound replacing U-46619 as a standard agonist. Indeed, of the thromboxane A_2 mimetics we have examined, U-46619 has the most favourable combination of potency, specificity and rapidity of action. Nevertheless the mechanisms underlying the slow kinetics of EP 171 action are of considerable interest. The ligand binding experiments indicate a K_i for EP 171 of 2.9 nM. The true K_i may be somewhat lower for two reasons. First, EP 171 is racemic and it is possible that only one enantiomer competes effectively with the radioligand in the binding assay. In the case of the parent compound, the isomer formally related to PGH_2/TXA_2 is about 100 times more potent than its mirror image as an activator of human platelets (Sprague *et al.*, 1985). Secondly, some reduction in the initial free EP 171 concentration may occur due to TP-receptor binding. Assuming that each platelet possesses 1700 TP-receptors (Armstrong *et al.*, 1983) the concentration of TP-receptors will be about 0.7 nM. The IC_{50} value of 1.45 nM for the active species of EP 171 would therefore be reduced to about 1 nM (the initial free concentration of the less potent competing ligands is likely to be reduced by less than 10%). Could an equilibrium dissociation constant of about 1 nM account for the slow kinetics of EP 171 action? Let us deal with the organisationally simpler platelet system first. The antagonist-t_{off} value for EP 171 of about 110 s (Figure 6c) will reduce to 70 s when EP 171 concentration is substituted for response magnitude (a reduction in shape change response from 80% to 40% of maximum corresponds to a 3.0 fold reduction in EP 171 concentration). A t_{off} of 70 s corresponds to a dissociation rate constant (k_2) for the EP 171/TP-receptor interation of 0.01 s^{-1}, and an association rate constant (k_1) of 1×10^7 $M^{-1}s^{-1}$ derives from a K_d of 1 nM. The rate constant for receptor occupation (assuming no reduction in initial free ligand concentration) is given by $k_1[A] + k_2$ (Paton & Rang, 1965). At low concentrations of EP 171 (0.1 nM or less) the term $k_1[A]$ becomes negligible and the rate of receptor occupation is dependent only on k_2. The half-times for onset and offset of EP 171 action should therefore be very similar. However, t_{on} for EP 171 is clearly less than t_{off} (Figure 6c). The discrepancy may lie in an overestimation of t_{off}, since we have observed that the rate of reversal of shape change produced by a fast acting agonist (e.g. U-46619) declines if the agonist is allowed more than 3 min contact before the TP-receptor antagonist is added. In addition, the shape change becomes more difficult to reverse if the platelets are used more than 2 h after PGI_2 decay. It is possible that the intracellular events associated with TP-receptor agonist-induced shape change tend to lose some of their ability to reverse during prolonged agonist contact.

The suggested magnitude of k_2 for the EP 171/TP-receptor interaction is small for an agonist/receptor interaction (see Ginneken, 1977) but not for an antagonist-receptor interaction. For example, the best non-ligand binding estimate of the rate constants for the atropine/muscarinic receptor interaction is probably that of Bolton (1977) using iontophoretic application of drugs to guinea-pig taenia coli: $k_1 = 1 \times 10^7 M^{-1}s^{-1}$, $k_2 = 0.011 s^{-1}$ and $K_d = 1.1$ nM. Ligand binding experiments with radiolabelled EP 171 could provide information on the magnitudes of k_1 and k_2. However, the radioligand would require to have a high specific activity since the free ligand concentration would be in the 1–10 nM range. The simple expedient of partially

replacing the 15β-proton with tritium (Armstrong *et al.*, 1983) may not be sufficient for this purpose.

The t_{on} and t_{off} values obtained on the rabbit aorta and guinea-pig trachea are considerably greater than those found for the human platelet system, although similar trends are seen. To obtain half-times related to agonist concentration the values shown in Figure 6a, b must be divided by 1.55 for both rabbit aorta and guinea-pig trachea. It seems unlikely that half-times in excess of 60 min simply reflect the rate constant for occupation of TP-receptors by EP 171 and one must look for mechanisms whereby the access of agonist to cells in the centre of the tissue is restricted. Factors potentially controlling the rate of drug action in densely packed tissues such as smooth muscle or nerves fibres have been considered by several workers (Furchgott, 1964; Rang, 1966; Colquhoun & Ritchie, 1972; Colquhoun *et al.*, 1972). In the exact diffusion equation approach of Colquhoun *et al.* (1972) it is suggested that diffusion of the drug in and out of the tissue is rate-limiting, with equilibration at receptors being relatively rapid. Diffusion of drug through the extracellular fluid (e.c.f.) is slowed by one or more cell membrane-based processes which abstract drug molecules from the e.c.f. These processes could include saturable binding to cell surface receptors, active uptake into the cell and passive transfer into lipophilic areas of the cell. In the case of saturable binding the diffusion coefficient is reduced by a factor $1 + M/K_d V$, when the drug concentration is much smaller than K_d (M is the binding capacity and V the volume of the extracellular space). For tetrodotoxin (TTX) binding ($K_d = 3$ nM) to the desheathed rabbit vagus nerve it was suggested that the above mechanism could slow the rate of equilibration of TTX by more than a thousand fold. If the K_d of EP 171 binding to TP-receptors in smooth muscle is similar to that determined for human platelets, then this mechanism rather than sequestration into lipid control also account for the slow kinetics of EP 171 action; the parameter we are lacking to complete the comparison with the TTX data is the TP-receptor binding capacity of the smooth muscle systems. It is of interest that in a quite distinct system, steroid inotropic activity on guinea-pig papillary muscle, the rate of onset of action decreased as potency of the analogue increased (Ebner, 1987). It was also shown that the rate of onset decreased as aqueous diffusion distance (muscle diameter) increased, and that this was most marked with highly potent steroids (e.g. digitoxin). The author favoured reduced diffusion through the e.c.f. due to binding to steroid receptors rather than lipophilic uptake into the myocytes to account for these findings.

On the rabbit aorta the half-time for EP 171 offset due to TP-receptor blockade was much smaller than the half-time for offset due to washing (Figure 6a). However, on the guinea-pig trachea the difference was less striking (Figure 6b) and the EP 171 contraction decayed slowly ($t_{off} \sim 40$ min) even with a high concentration (10 μM) of EP 092 (predicted dose-ratio = 1000). More limited observations with two other potent TP-receptor antagonists, GR 32191 (10 μM) and ONO 11120 (5 μM), gave similar profiles. A somewhat different situation from that found with EP 171 on the guinea-pig trachea is seen with salmeterol, a salbutamol analogue in which a 11-phenyl-6-oxaundecyl group replaces the *t*-butyl group (Bradshaw *et al.*, 1987). Salmeterol activates β_2-adrenoceptors to produce tracheal relaxation which is very slowly reversed by washing. However, addition of the β-blocker propranolol rapidly reverses the inhibition (Ball *et al.*, 1987). Further investigations are obviously required to ascertain whether the slow offset of EP 171 action due to receptor blockade can be mainly attributed to its high affinity for the TP-receptor.

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