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RESEARCH PAPER

Interaction of prostanoid EP_3 and TP receptors in guinea-pig isolated aorta: contractile self-synergism of 11-deoxy-16,16-dimethyl PGE_2

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BACKGROUND AND PURPOSE
Surprisingly high contractile activity was reported for 11-deoxy-16,16-dimethyl prostaglandin E_2 (DX-DM PGE_2) on pig cerebral artery when used as a selective EP_3 receptor agonist. This study investigated the selectivity profile of DX-DM PGE_2, focusing on the interaction between its EP_3 and TP (thromboxane A_2-like) agonist activities.

EXPERIMENTAL APPROACH
Contraction of guinea-pig trachea (EP_1 system) and aorta (EP_3 and TP systems) was measured in conventional organ baths.

KEY RESULTS
Strong contraction of guinea-pig aorta to sulprostone and 17-phenyl PGE_2 (EP_3 agonists) was only seen under priming with a second contractile agent such as phenylephrine, histamine or U-46619 (TP agonist). In contrast, DX-DM PGE_2 induced strong contraction, which on the basis of treatment with (DG)-3ap (EP_3 antagonist) and/or BMS-180291 (TP antagonist) was attributed to self-synergism arising from co-activation of EP_3 and TP receptors. EP_3/TP self-synergism also accounted for contraction induced by $PGF_{2\alpha}$ and its analogues (+)-cloprostenol and latanoprost-FA. DX-DM PGE_2 also showed significant EP_1 agonism on guinea-pig trachea as defined by the EP_1 antagonists SC-51322, (ONO)-5-methyl-1 and AH-6809, although AH-6809 exhibited poor specificity at concentrations $\geq$3 μM.

CONCLUSIONS AND IMPLICATIONS
EP_3/TP self-synergism, as seen with PGE/PGF analogues in this study, may confound EP_3 agonist potency comparisons and the characterization of prostanoid receptor systems. The competitive profile of a TP antagonist may be distorted by variation in the silent/overt contraction profile of the EP_3 system in different studies. The relevance of self-synergism to *in vivo* actions of natural prostanoid receptor agonists is discussed.

Abbreviations
FA, free acid form of a C1-ester or C1-amide prostanoid; n_H, Hill slope for a sigmoidal log concentration–response curve; pA_2, negative logarithm of the molar concentration of antagonist producing a dose ratio of 2; pEC_{50}, negative logarithm of the agonist concentration inducing 50% maximum response; PGE_2, prostaglandin E_2; pIC_{50}, negative logarithm of the molar concentration producing 50% inhibition of an established response; TXA_2, thromboxane A_2

Introduction

Prostaglandin E_2 (PGE_2) has a broad range of actions including excitation of smooth muscle cells mediated by prostanoid EP_1 and EP_3 receptors and inhibition mediated by EP_2 and EP_4 subtypes (see Coleman *et al.*, 1994; receptor nomenclature follows Alexander *et al.*, 2009). Its contractile action on pig

isolated cerebral artery has been linked to activation of both EP_1 and EP_3 receptors operating through the phosphatidyl inositol pathway (Jadhav *et al.*, 2004). We had two major concerns about this study. First, very high concentrations (30–300 μM) of the EP_1 antagonist AH-6809 (Coleman *et al.*, 1985; 1987) were used to identify the EP_1 component and also to gauge the selectivity of 11-deoxy-16,16-dimethyl PGE_2 (DX-DM PGE_2), which was used as a selective EP_3 agonist. Second, DX-DM PGE_2 was about 50 times more potent and had a higher maximum response than sulprostone, a potent full agonist for the EP_3 receptor (Lawrence *et al.*, 1992; Sharif *et al.*, 1998). Given that pig cerebral artery contains a contractile TP receptor system (Wallis and Martin, 2000) and other 11-deoxy PGE and PGF analogues show considerable TP agonism (Jones *et al.*, 1982; Banerjee *et al.*, 1985), the remarkably high activity of DX-DM PGE_2 may reflect simultaneous activation of EP_3 and TP receptors.

It was decided therefore to examine the profile of DX-DM PGE_2 on guinea-pig isolated trachea, which contains a contractile EP_1 system (Jones *et al.*, 1982; Coleman and Kennedy, 1985) and guinea-pig aorta, which contains EP_3 and TP contractile systems (Jones *et al.*, 1998; 2002). AH-6809 and two chemically distinct EP_1 receptor antagonists, SC-51322 (Hallinan *et al.*, 1994) and (ONO)-5-methyl-1 (Jones *et al.*, 2010) were used to block EP_1 receptors. EP_3 receptors were blocked by the acyl-sulphonamide (DG)-3ap, which achieves steady-state antagonism faster than its relatives L-798106 and L-826266 (Gallant *et al.*, 2002; Belley *et al.*, 2005; Schlemper *et al.*, 2005) possibly due to its lower lipophilicity (Jones *et al.*, 2010). TP receptors were blocked with BMS-180291, chosen for its high affinity (pA_2 = 9.8, Zhang *et al.*, 1996).

Unexpectedly, sulprostone induced minimal contraction of the guinea-pig aorta preparation; there was however powerful synergism with strong contractile agents, including the TP receptor agonist U-46619. The potential for DX-DM PGE_2 to self-synergize through co-activation of EP_3 and TP receptors therefore became a major focus of the investigation. Synergism profiles were also determined for $PGF_{2\alpha}$ and two of its analogues (+)-cloprostenol and latanoprost-FA, the last being a selective FP receptor agonist (see Jones *et al.*, 2009).

Our experiments have revealed that DX-DM PGE_2 potently activates EP_3 and TP receptors in guinea-pig aorta, resulting in contractile self-synergism. EP_3/TP self-synergism also accounts for the contractile activities of the FP receptor agonists examined. Moreover, variation in the silent/overt contractility of the EP_3 receptor system may distort the competitive profile of a TP antagonist owing to the EP_3 activity of the TP receptor agonist at high concentration. DX-DM PGE_2 is also a moderately potent EP_1 receptor agonist. In terms of EP_1 receptor antagonism, AH-6809 is much less specific than SC-51322 and (ONO)-5-methyl-1, suppressing the contractile activity of prostanoid and non-prostanoid agonists on trachea and aorta at concentrations of 3 μM and above. These findings have important implications for the characterization of prostanoid receptor systems. The role of self-synergism in the *in vivo* actions of natural prostanoid receptor agonists is discussed.

Methods

Isolated smooth muscle preparations

All animal care and experimental procedures were in compliance with the UK Animals (Scientific Procedures) Act 1986. Cervical trachea and descending thoracic aorta were dissected from male Dunkin-Hartley guinea-pigs (400–500 g, Harlan, UK) after killing by exposure to CO_2. Four contiguous rings, 4 mm in length, were suspended between L-shaped stainless steel wire holders, one of which was attached to a Grass FT03 transducer, in conventional 10 mL tissue baths. The isometric tension signal was relayed to a AD Instruments PowerLab preamplifier-digitizer/Dell computer system. The bathing fluid was Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.18 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose) aerated with 95% O_2/5% CO_2, maintained at 37°C, and containing 1 μM indomethacin to inhibit cyclo-oxygenase activity. Resting tension was adjusted to 1.2 g for both preparations. Endothelium was removed from some aorta preparations by gentle rotation of the vessel ring on a wooden cocktail stick (Jones *et al.*, 1998); this procedure abolished relaxation induced by 3 μM acetylcholine against established contraction to 2 μM phenylephrine, without affecting similar relaxation induced by 3 nM cicaprost (IP receptor agonist).

Experimental protocols

Cumulative addition of compounds was routinely performed. Initial sequences were: trachea: 40 mM K^+; 1, 3, 10 nM 17-phenyl PGE_2, aorta: 40 mM K^+; 1, 5 μM phenylephrine.

Agonist protocols on trachea: 3–4 prostanoid agonist sequences were obtained at 2 h intervals in the presence of 1 μM BMS-180291 (45 min pretreatment). A full concentration–response curve to carbachol (10–1000 nM) was obtained at the end of the experiment.

Antagonist inhibition-curve protocols on trachea and aorta. After establishment of a E_{70} response to a particular agonist (with priming as necessary), a series of antagonist doses was added. K^+ was applied without any correction for increase in ionic strength.

Antagonism pretreatment protocols on aorta. The following sequences were applied: 10 nM 17-phenyl PGE_2 under 10 nM U-46619 priming (90 min after set-up); 1, 3, 10 μM phenylephrine (190 min); antagonist (TP, EP_3, TP + EP_3) or vehicle (225 min) followed by a prostanoid agonist sequence under phenylephrine priming (270 min). Preparations were then washed for 90 min in the presence of the EP_3/TP receptor antagonist combination before a full curve to phenylephrine (0.3–30 μM) was obtained. Precise time-matching was essential in each experiment owing to the gradual increase in the maximum responses of strong agonists (typically 50–70% for phenylephrine, histamine and U-46619) throughout the experimental period (6 h); small increases in corresponding pEC_{50} values also occurred (0.1–0.2 log units).

Data analysis

Contractile responses were measured from the resting level and normalized to the carbachol and phenylephrine maximum responses on trachea and aorta respectively. Log concentration–response curves were fitted by a four-parameter (variable slope) sigmoidal curve with constraint of the lower asymptote to resting tone/priming level/E_{70} as appropriate (GraphPad Prism software, La Jolla, CA, USA). Theoretical sigmoidal curves were constructed using the same software. pA_2 values were calculated by substitution of dose ratios into the Gaddum-Schild equation: $pA_2 = \log(\text{dose ratio} - 1) - \log[\text{antagonist}]$. Dunnett's multiple comparison test (GraphPad Prism) was applied to data from the antagonist pretreatment experiments (control = vehicle treatment); the significance level was set at $P = 0.05$. The error associated with a mean value is the s.e.mean.

Materials

Stock solutions (10 mM) of prostanoid ligands and other compounds were prepared in absolute ethanol and water, respectively, unless stated otherwise. Dilutions were prepared with 0.9% NaCl solution (saline); the first dilutions of AH-6809, (DG)-3ap and U-46619 were solubilized with a trace of $NaHCO_3$. Sources of prostanoid ligands: Allergan, USA, cicaprost, (ONO)-5-methyl-1 ({[2-[isobutyl(phenylsulphonyl)amino]-5-(methyl)phenoxy]methyl}benzoic acid; 5-methyl derivative of compound 1 described by Naganawa *et al.* (2006), 10 mM in DMSO): Biomol International, UK, SC-51322 (8-chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-[3[2-(furanylmethyl)thio]1-oxopropyl]hydrazine, 10 mM in DMSO): Bristol-Myers Squibb, USA; BMS-180291 ([1*S*-(*exo*,*exo*)]-2-[[3-[4-[(pentylamino)carbonyl]-2-oxazolyl]-7-oxabicyclo[2.2.1]hept-2-yl]methyl]-benzenepropanoic acid, Ifetroban): Cayman Chemical, USA; AH-6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid), (+)-cloprostenol, I-BOP ([1S-(1a,2b(5*Z*),3a(1*E*,3*S*),4a]-7-[3-(3-hydroxy-4-(4′-iodophenoxy)-1-butenyl)-7-oxabicyclo-[2.2.1]heptan-2-yl]-5-heptenoic acid, 0.2 mM in ethanol), latanoprost-FA (PhXA 85), $PGF_{2\alpha}$, 17-phenyl-ω-trinor PGE_2, U-46619 (15*S*-hydroxy-11α,9α-epoxymethano-prosta-5*Z*,13*E*-dienoic acid): Target Molecules, UK; (DG)-3ap (1-(3-methoxybenzyl)-3a-methyl-[3,3a,4,5,6-hexahydroindol-2-one-7-acrylic acid, 3,4-difluorobenzenesulphonamide, compound 3ap described by O'Connell *et al.* (2009), 10 mM in DMSO). Sources of other compounds: Fluka Chemical, Switzerland: phenylephrine hydrochloride, carbachol chloride; Sigma-Aldrich, USA: acetylcholine chloride, indomethacin (20 mM in ethanol).

Results

Selective block of EP_1 and EP_3 receptors

Concentrations of antagonists used to selectively block EP_1 and EP_3 receptors in subsequent experiments were determined by cumulative inhibition of an established E_{70} response (inhibition-curve protocol). On guinea-pig trachea, EP_1, TP and muscarinic M_3 receptor-mediated contractions (typically 2.5–3.5 g) were induced with 17-phenyl PGE_2 (3–5 nM), U-46619 (20–30 nM) and carbachol (50–60 nM) respectively. On the guinea-pig aorta, the EP_3 response was elicited by 17-phenyl PGE_2 (25 nM) under priming with the α_1-adrenoceptor agonist phenylephrine (0.3–1.0 μM, cf. Figure 1C). 17-Phenyl PGE_2 was preferred to the more potent EP_3 agonist sulprostone (3 nM) owing to its faster onset and offset of action (see Jones *et al.*, 2010 for further details). Although 17-phenyl PGE_2 responses were enhanced under threshold priming (<5% of phenylephrine maximum), priming to 20–25% afforded less variation in the EP_3 maximum response both within and between experiments, and also allowed the effects of prostanoid antagonists on the priming response to be accurately assessed (see later). Higher phenylephrine priming (40–50%) often resulted in fading of EP_3 responses greater than E_{60}. The TP receptor antagonist BMS-180291 (1 μM), which had no effect on established 17-phenyl PGE_2 responses on trachea (not shown) and aorta (Figure 1C), was present throughout the EP_1 and EP_3 inhibition-curve protocols.

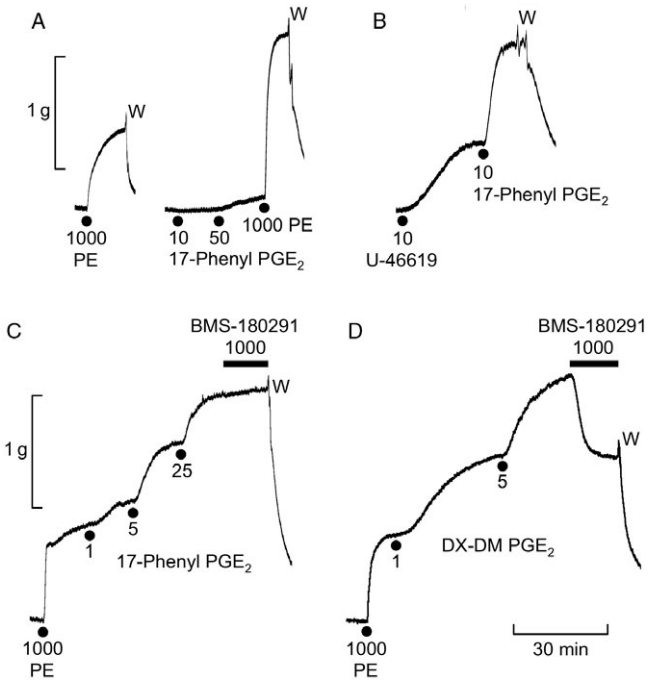

Figure 1

Experimental records illustrating properties of the EP_3 receptor contractile system in guinea-pig isolated aorta. (A) Weak contraction/enhancement of the response to phenylephrine (PE) induced by the EP_3 standard agonist 17-phenyl PGE_2. (B) Enhanced response to 17-phenyl PGE_2 under priming with the TP receptor agonist U-46619. (C) Enhanced responses to 17-phenyl PGE_2 under phenylephrine priming and nil effect of the TP antagonist BMS-180291 on the established contraction. (D) Partial inhibition of phenylephrine-primed DX-DM PGE_2 contraction by BMS-180291. W = wash. Concentrations in nM.

High EP_1 receptor selectivity was shown by SC-51322 (pIC_{50} = 7.26 ± 0.10, n = 4), with <10% inhibition of EP_3, TP and M_3 receptor agonism at 10 μM (Figure 2). The EP_1 receptor antagonism of (ONO)-5-methyl-1 was slow to reach steady state and cumulative concentrations of 5 and 15 nM only were applied; it was about nine times more potent than SC-51322 (pIC_{50} ~8.2). At 1–3 μM, it significantly inhibited EP_3 and TP responses, while M_3 agonism was essentially unaffected. AH-6809 was the least potent (pIC_{50} = 6.78 ± 0.03 on trachea) and least selective EP_1 antagonist. At 3 and 10 μM, it significantly inhibited TP and M_3 receptor-mediated contraction on trachea, and also EP_3, TP, α_1-adrenoceptor and histamine H_1 receptor agonsim on aorta (inset in Figure 2C, only 10 μM data shown); inhibitions at 1 μM AH-6909 were all minimal. SC-51322, (ONO)-5-methyl-1 and AH-6809 concentrations of 1.0, 0.1 and 1.0 μM, respectively, were considered suitable for selective EP_1 receptor blockade

The net EP_3 response of guinea-pig aorta induced by 25 nM 17-phenyl PGE_2 was not completely inhibited by (DG)-3ap (pIC_{50} ~7.0, Figure 2D), possibly due to residual synergism maintained by α_1 agonism (priming) alone (Jones *et al.*, 2010). Inhibition of EP_1 and TP responses by (DG)-3ap on trachea was significant (pIC_{50} = 5.73 ± 0.08 and 5.35 ± 0.09, respectively), while suppression of M_3-receptor responses was minimal. A (DG)-3ap concentration of 1 μM was chosen for selective EP_3 receptor blockade. In the presence of 1 μM (DG)-3ap, established contraction of the aorta to a 20-fold higher concentration of 17-phenyl PGE_2 (500 nM) was unaffected by addition of the three EP_1 receptor antagonists at the chosen concentrations (n = 3, data not shown).

Agonist profiles of PGE and PGF analogues on guinea-pig trachea and aorta

In terms of EP_1 receptor agonism on guinea-pig trachea, 17-phenyl PGE_2 (standard agonist), DX-DM PGE_2 and (+)-cloprostenol exhibited parallel log concentration–response curves with pEC_{50} values of 8.79 ± 0.08 (n = 8), 6.81 ± 0.13 (n = 4) and 5.85 ± 0.12 (n = 4) respectively (1 μM BMS-180291 present). The corresponding equi-effective molar ratios are 1.0, 95 and 870. Established EC_{70} responses to DX-DM PGE_2 and (+)-cloprostenol were abolished by the chosen concentrations of the three EP_1 receptor antagonists (all n = 3).

In relation to EP_3 receptor agonism on guinea-pig aorta, DX-DM PGE_2, $PGF_{2\alpha}$ (+)-cloprostenol and latanoprost-FA differed radically from 17-phenyl PGE_2 (and sulprostone) in inducing strong contraction under resting tone; pEC_{50} values were 8.25 ± 0.17, 6.58 ± 0.07, 6.55 ± 0.05 and 5.25 ± 0.15 respectively (n = 4). Moreover, 1 μM BMS-180291 partially inhibited their phenylephrine-primed E_{70} responses, typically by 25–50% (see Figure 1D for DX-DM PGE_2). Contraction induced by 17-phenyl PGE_2 was markedly enhanced by priming with U-46619 (5–10 nM, Figure 1B). It thus appeared that DX-DM PGE_2 and the PGF analogues might activate both EP_3 and TP receptors resulting in a pronounced contractile synergism. Enhancement of EP_3 receptor agonism was also seen under priming with histamine (1 μM, H_1 agonist) or K^+ (20–25 mM) (data not shown). Removal of the aortic endothelium by gentle abrasion did not significantly alter the agonist profiles of 17-phenyl PGE_2 and DX-DM PGE_2 shown in Figure 1 (data not shown).

Effect of EP_3/TP receptor antagonist pretreatment on primed responses to PGE and PGF analogues on guinea-pig aorta

It was considered useful to obtain separate EP_3 and TP receptor concentration–response profiles for DX-DM PGE_2 and the three PGF analogues on guinea-pig aorta under the same degree of priming.

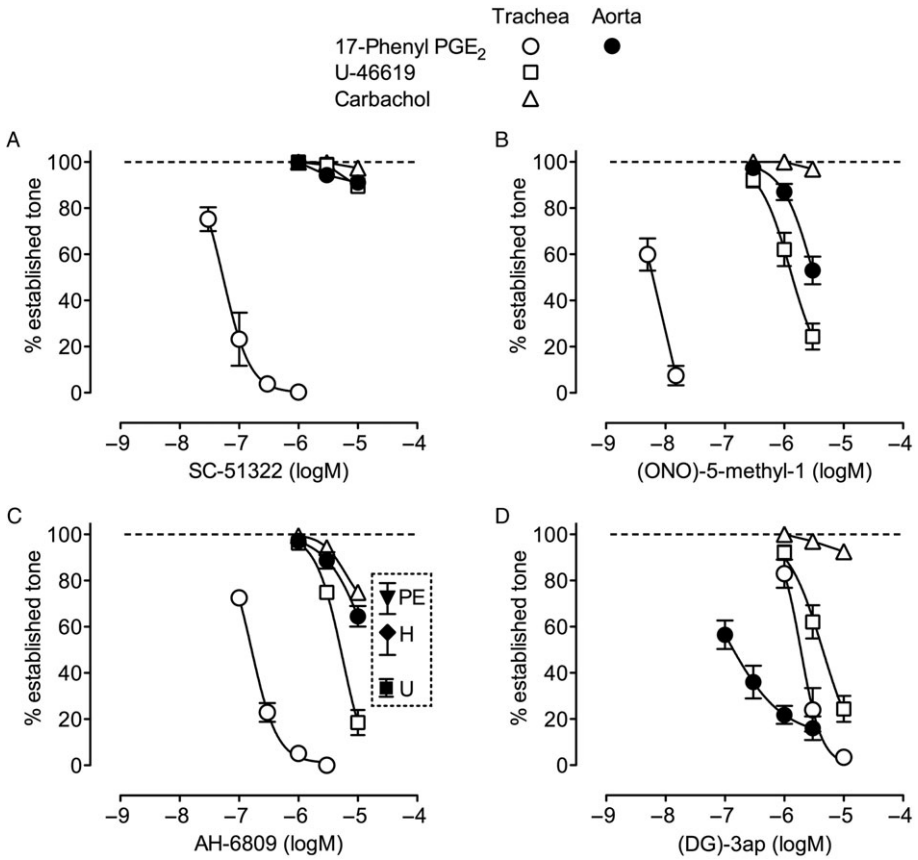

Figure 2

Selectivity of EP receptor antagonists: cumulative inhibition curves for (A) SC-51322 (B) (ONO)-5-methyl-1 (C) AH-6809 and (D) (DG)-3ap on guinea-pig trachea and aorta. Contraction was established with 17-phenyl PGE_2, U-46619 and carbachol on trachea and 17-phenyl PGE_2 primed with phenylephrine (net EP_3 response = 100%) on aorta. The inset in panel C shows the effect of 10 μM AH-6809 against phenylephrine (2 μM, PE), histamine (1.5 μM, H) and U-46619 (20–30 nM, U) on guinea-pig aorta; inhibition with 1 μM AH-6809 was <5% in each case. BMS-180291 (1 μM) was present for all 17-phenyl PGE_2 tests. Error bars indicate s.e.mean (n = 4). Carbachol activates M_3 receptors in guinea-pig trachea (Morrison and Vanhoutte, 1992).

Preparations were therefore pretreated with vehicle, 1 μM (DG)-3ap, 1 μM BMS-180291 and a combination of the antagonists for 45 min before prostanoid agonist was applied cumulatively under phenylephrine priming (Figure 3). Statistical analysis using Dunnett's multiple comparison test showed that, for each prostanoid agonist examined, the initial TP and EP_3 receptor agonist sensitivities of the four groups of aorta preparations were uniform: no significant differences ($P > 0.05$) between responses to 10 nM U-46619 and between incremental (primed) responses to 10 nM 17-phenyl PGE_2 (cf. Figure 1B). Using the same analysis, phenylephrine priming responses in the presence of the antagonist treatments were not significantly different to the vehicle control ($P > 0.05$). A log concentration–response curve for 17-phenyl PGE_2 (0.5–312.5 nM) in the presence of 1 μM BMS-180291 obtained in contemporaneous experiments (n = 10) is also shown in Figure 3B; fitting parameters (mean, 95% CI) were: priming level = 21.5% (18–25), maximum = 78% (73.5–82.5), pEC_{50} = 8.42 (8.28–8.55), n_H = 0.77 (0.71–0.84).

Under TP receptor blockade, maximum responses to DX-DM PGE_2, PGF_{2a} (data not shown) and (+)-cloprostenol approach 80% of the phenylephrine maximum, similar to 17-phenyl PGE_2 and indicative of activation of EP_3 receptors. This is supported by the rightward shifts caused by 1 μM (DG)-3ap (in the presence of BMS-180291); corresponding pA_2 values are 7.96 ± 0.07, 7.55 ± 0.03 and 8.15 ± 0.03, which are similar to the value of 7.92 ± 0.07 obtained with 17-phenyl PGE_2 as agonist (Schild plot, Jones *et al.*, 2010). A similar analysis was not possible for latanoprost-FA owing to its low potency. Nevertheless, there was a clear suppression of its contractile action by (DG)-3ap in the presence of BMS-180291. Under EP_3 receptor blockade, BMS-180291 treatment revealed TP receptor agonism for each analogue. Contraction induced by

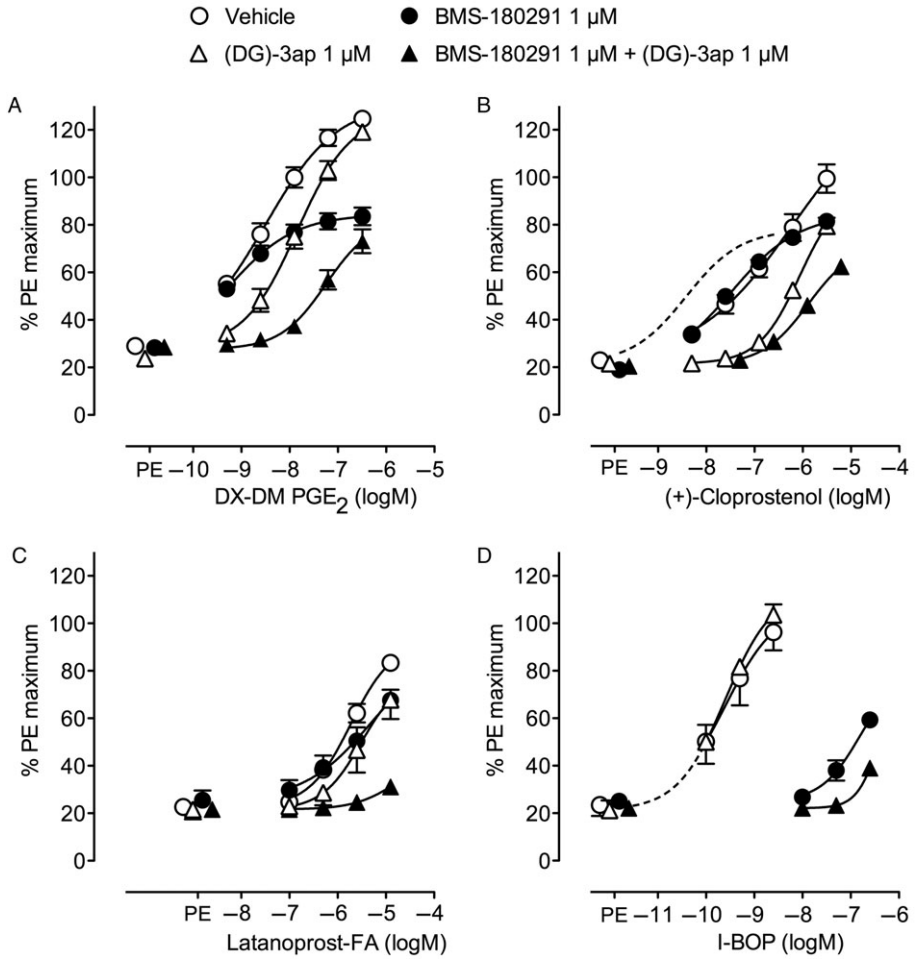

Figure 3

Log concentration–response curves for prostanoid agonists on guinea-pig aorta under phenylephrine (PE) priming in the presence of vehicle, BMS-180291, (DG)-3ap and a combination of BMS-180291 and (DG)-3ap: (A) DX-DM PGE_2 (B) (+)-cloprostenol (C) latanoprost-FA and (D) I-BOP. Responses were normalized to the maximum response to phenylephrine on each preparation in the presence of BMS-180291 + (DG)-3ap. Error bars indicate s.e.mean (n = 4). The broken line in B is the fitted curve for 17-phenyl PGE_2 in the presence of 1 μM BMS-180291 obtained in contemporaneous experiments (mean priming = 21.5%, n = 10). The broken line in D is an extrapolation of the I-BOP/1 μM (DG)-3ap curve.

the potent TP receptor agonist I-BOP (Sessa *et al.*, 1990; Matsuda *et al.*, 1994) was particularly slow and it was only possible to challenge with three mid-range doses; responses to the two lower doses may not have reached steady state. (DG)-3ap did not block I-BOP-induced contraction, attesting to its selectivity in this situation. It is likely that the response to the highest concentration of I-BOP (250 nM) in the presence of BMS-180291 + (DG)-3ap contains a TP component owing to I-BOP overcoming the BMS-180291 blockade: dose ratio for EP_3 + TP versus EP_3 treatment at 35% phenylephrine maximum level is ~5900; dose ratio calculated for 1 μM BMS-180291 using pA_2 of 9.98 obtained in a concurrent study is 9550 (Schild plot, U-46619 as agonist, Jones *et al.*, 2010).

For easier appreciation, the data in Figure 3 have been presented as EP_3 and TP receptor activation ranges (Figure 4). Each range corresponds to E_3 – E_{97} (obtained by extrapolation as necessary); the range for an agonist with n_H of 1.0 covers 3.0 log units. Estimation of EP_3 activation ranges (~4.0 log units, n_H ~0.75) is straightforward because of the comprehensive block of TP receptors by BMS-180291. The TP receptor activity ranges for I-BOP and U-46619 cover ~3.5 log units (n_H ~0.87), but are less well-defined for the other prostanoid agonists given the weaker block produced by (DG)-3ap, and this applies particularly to (+)-cloprostenol. The EC_{50} values for the prostanoid agonists acting alone have also been included.

Further investigation of the antagonist profile of BMS-180291 on guinea-pig aorta

Using a conventional Schild protocol, Zhang *et al.* (1996) obtained a pA_2 value of 9.8 for BMS-180291 versus U-46619 (pEC_{50} ~8.0) on guinea-pig aorta, but

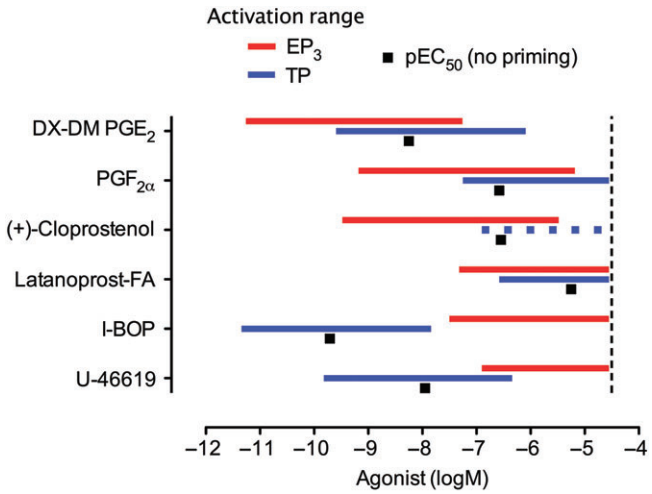

Figure 4

EP_3 and TP receptor activation ranges for prostanoid agonists under phenylephrine priming on guinea-pig aorta. Each horizontal bar represents the concentration range corresponding to E_3 – E_{97}; the broken bar for (+)-cloprostenol indicates difficulty in estimation, because of interference from the other receptor activity. The black squares indicate pEC_{50} values in the absence of priming; the I-BOP data point derives from Jones *et al.* (2010) in which steady-state responses were obtained by applying single concentrations of I-BOP to multiple preparations. The vertical line represents a nominal upper limit of 30 μM.

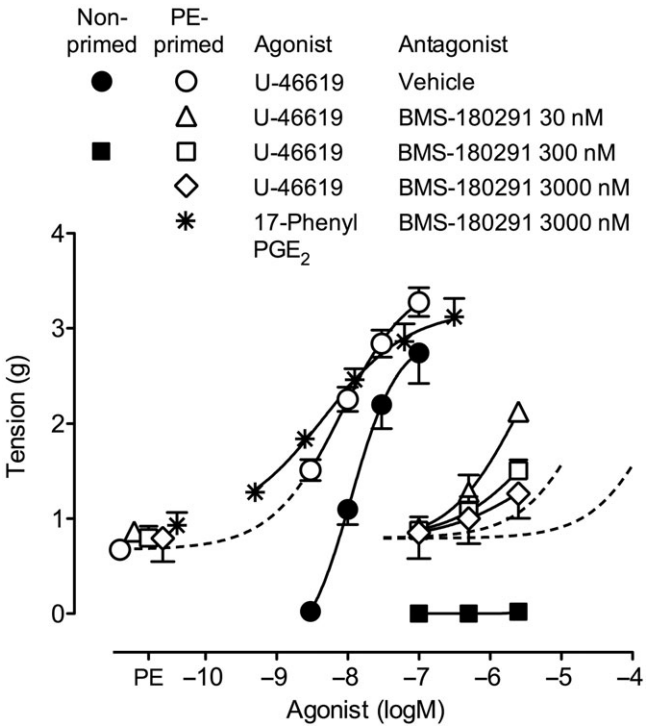

Figure 5

Guinea-pig aorta: effect of the TP receptor antagonist BMS-180291 on log concentration–response curves for U-46619 with and without phenylephrine (PE) priming. The broken line curves on the right are predicted for 300 and 3000 nM BMS-180291 assuming a pA_2 of 9.98 (taken from Jones *et al.*, 2010) and n_H of 0.87 for U-46619. The curve for PE-primed 17-phenyl PGE_2 in the presence of 3000 nM BMS-180291 is also shown. Error bars indicate s.e.mean (n = 4).

failed to produce dose ratios in excess of ~20. This was not the case in the current study, where pretreatment with 300 nM BMS-180291 for 60 min essentially abolished the response to 2.5 μM U-46619 (Figure 5). Furthermore, under phenylephrine priming, 30 nM BMS-180291 afforded a U-46619 dose ratio of ~295 (pA_2 = 9.99). BMS-180291 at 300 nM and 3 μM produced larger rightward shifts, but these were less than expected for competitive antagonism according to the Schild equation (see broken curves in Figure 5). The residual response to 2.5 μM U-46619 in the presence of 3 μM BMS-180291 was abolished following addition of 1 μM (DG)-3ap (data not shown), indicating activation of EP_3 receptors by U-46619. High sensitivity to 17-phenyl PGE_2 was maintained in the presence of 3 μM BMS-180291 (pEC_{50} = 8.33 ± 0.03, n = 4, Figure 5).

Discussion

EP_3 receptor-mediated contractility on guinea-pig aorta

EP_3 receptor agonism on guinea-pig aorta was characterized by weak overt contraction accompanied by pronounced synergism with a second strong contractile agent such as phenylephrine (selective α_1-adrenoceptor agonist), U-46619 (TP receptor agonist) or K^+ (membrane-depolarizing agent). We presume that these interactions primarily involve smooth muscle cells as contraction profiles were not affected by endothelium removal. Activation of EP_3 receptors located on post-synaptic sympathetic neurones in rat and human blood vessel preparations results in suppression of transmitter release (Molderings *et al.*, 1992; 1994); it is not known whether an analogous action influenced the contractility profiles found in this study. Low maximum/high synergy agonist profiles have been previously described for 5-HT_{1B}, α_2-adrenoceptor and neuropeptide Y Y_1 receptor systems in vascular smooth muscle (De La Lande *et al.*, 1966; Edvinsson *et al.*, 1984; Wahlestedt *et al.*, 1985; McGrath *et al.*, 1990); the corresponding agonists are often described as 'silent'. Yildiz *et al.* (1998) have reviewed the discrimination of this marked contractile synergism from 'mutual effect amplification', in which a small

degree of synergism arises when two agonists activate receptors (e.g. α_1-adrenoceptor/H_1 receptor) utilizing the same transduction system(s) (Leff, 1987; Christ and Jean-Jacques, 1991). They further distinguished inertial (threshold) synergism, whereby 'a certain amount of stimulus must be delivered in order for the tissue to approach the threshold for contraction' (Ariens *et al.*, 1960; Stupecky *et al.*, 1986).

In contrast to the current study, one of us previously found that EP_3 receptor agonists induced much stronger contraction of guinea-pig aorta (30–60% of the tissue maximum; synergism with phenylephrine or U-46619 was moderate (Jones *et al.*, 1998; 2002). The animals (Dunkin-Hartley strain) in the two situations came from unrelated breeding colonies. Studies in isolated cell systems show that EP_3 receptors exist in several isoforms (Sugimoto and Narumiya, 2007). All EP_3 isoforms couple efficiently to Gi (as do 5-HT_{1B}, α_2-adrenoceptor and neuropeptide Y Y_1 receptor subtypes; see Yildiz *et al.*, 1998), while they differ in their abilities to couple to Gq and Gs; their agonist recognition profiles are similar. Thus it is possible that the different contractility profiles are due to variation in the proportions of EP_3 receptor isoforms present and/or the amplifications of their transduction systems. Higher overt contraction could be due to a more prominent Gq (PLC)-driven component of signal transduction. Alternatively, a silent profile could indicate operation of a significant Gs (cAMP)-driven component that suppresses contractile activity. In this context (post-synaptic) α_2-adrenoceptor systems in blood vessels from different species and peripheral locations vary in their need for priming to achieve strong contraction (see Blaylock and Wilson, 1995).

EP_3/TP receptor self-synergism on guinea-pig aorta

Our results indicate that strong contraction of guinea-pig aorta elicited by DX-DM PGE_2 and the PGF analogues may be attributed to co-activation of EP_3 and TP receptors resulting in a synergistic response, which we have termed self-synergism. The steep lower section of the log concentration–response curve for non-primed U-46619 on the aorta, which is poorly fitted by a symmetrical sigmoidal curve constrained to the resting tension (Figure 5), hints at inertial constraint of the TP receptor input. Priming with phenylephrine exposes excitation at lower U-46619 concentrations and we propose that this condition affords a truer picture of the TP receptor activation range. Thus for DX-DM PGE_2 at 1 nM, significant TP activation is accompanied by at least half maximal activation of the EP_3 system (Figure 4). A similar relationship is seen for $PGF_{2\alpha}$ and (+)-cloprostenol, while the EP_3 and TP inputs for latanoprost-FA overlap more. In contrast, I-BOP maximally activates the TP system before the EP_3 system is brought into play at around 30 nM. In this context, Kiriyama *et al.* (1997) reported binding Kds of 0.56 and 100 nM for I-BOP on mouse recombinant TP and EP_3 receptors, respectively; corresponding values for U-46619 were 67 and >10 000 nM. The potent and selective FP receptor agonist latanoprost-FA (Griffin *et al.*, 1997; Sharif *et al.*, 2002; Chen *et al.*, 2005) showed very low activity on the aorta in the presence of TP and EP_3 receptor blockade (Figure 3C), arguing against interference from a FP receptor system.

EP_1-selective concentrations of SC-51322, (ONO)-5-methyl-1 and AH-6809 failed to inhibit the modest response of the guinea-pig aorta to a high concentration of 17-phenyl PGE_2 under EP_3 receptor blockade with (DG)-3ap. Given that 17-phenyl PGE_2 is a potent EP_1 receptor agonist (Lawrence *et al.*, 1992), this protocol ought to have identified even a relatively insensitive EP_1 receptor system. Consequently, we propose that EP_1 agonism does not confound our analysis of the EP_3 receptor system in the aorta. This supposition may not apply to the Jadhav *et al.* (2004) study on pig cerebral artery, in which DX-DM PGE_2 was claimed to act as a selective EP_3 receptor agonist on the basis that its established contraction was not suppressed by 30–300 μM AH-6809. First, DX-DM PGE_2 at the high concentration used (3 μM) elicited near maximal contraction of the cerebral artery preparation. Second, this high concentration would be expected to cause considerable EP_1 (and TP) receptor activation based on our guinea-pig trachea (and aorta) data. Third, AH-6809 in high micromolar concentration may not specifically block EP_1 receptors. The low specificity of AH-6809 observed in our study (Figure 2C) may be due to functional antagonism driven by cAMP, as AH-6809 inhibited cAMP PDE in rat lung mast cells with IC_{50} of 26 μM (unpublished observations in Keery and Lumley, 1988). Correspondingly, 30 μM AH-6809 enhanced cAMP production by iloprost (IP receptor agonist) in washed platelet suspensions from man, rat and rabbit (Armstrong and Jones, 1988). So, *in toto*, strong (and potentially interactive) excitatory inputs from EP_1, EP_3 and TP receptors may have been opposed by both genuine EP_1 receptor antagonism and functional antagonism; a model of this complexity is fraught with interpretative dangers.

Relevance of self-synergism to prostanoid receptor characterization

On a simple level, it may not be realized that the relative potencies of agonists for the receptor of

interest are being distorted through self-synergism operating through a second receptor. Thus EP_3/TP self-synergism is a plausible explanation for the very high contractile activity of DX-DM PGE_2, relative to sulprostone or 17-phenyl PGE_2 in pig cerebral artery (Jadhav *et al.*, 2004). The confounding influence of self-synergism involving silent and overt contractile systems has been reported previously. For example, the selective α_2-adrenoceptor agonist rilmenidine enhanced the response to noradrenaline less than that to phenylephrine in rat tail artery, indicating that α_1/α_2-adrenoceptor self-synergism was already operating with the former non-selective agonist (Xiao and Rand, 1989). A similar explanation was advanced for the ability of angiotensin II (strong agonist) to enhance responses to the selective α_2 -adrenoceptor agonist UK-14304, but not to noradrenaline, in rabbit saphenous artery (Dunn *et al.*, 1991).

Taken to an extreme degree, there is the possibility of receptor misidentification. Cao *et al.* (2002) inferred the presence of a FP system in the non-pregnant pig uterus on the basis of the moderately potent contractile activity of the $PGF_{2\alpha}$ analogue cloprostenol (EC_{50} ~15 nM). However, the pig uterus preparation contains both EP_3 (Okada *et al.*, 2000) and TP (Cao *et al.*, 2004) contractile systems and, as we have shown on guinea-pig aorta (Figure 3B), cloprostenol [(+)-isomer] can achieve considerable contractile activity through EP_3/TP self-synergism. The judicious use of selective prostanoid receptor antagonists ought to clarify these situations.

In relation to characterizing TP antagonists, comprehensive block of TP receptors by BMS-180291 in the phenylephrine-primed guinea-pig aorta revealed EP_3 agonism for U-46619 at concentrations ≥500 nM, resulting in smaller right shifts than predicted for simple competition using the Schild equation (Figure 5). In a non-primed preparation, the potential EP_3/TP self-synergism of U-46619 is less likely to alter the competition profile as BMS-180291 will annul the TP receptor arm of the synergism. However, Zhang *et al.* (1996) reported that BMS-180291 failed to achieve dose ratios greater than 20 for antagonism of U-46619 in the non-primed guinea-pig aorta; addition of a second TP receptor antagonist (SQ-29548, 100 nM, predicted dose ratio = 35) did not cause a further right shift of the U-46619 curve. They proposed that U-46619 (≥200 nM) activated a second 'contractile' receptor. In view of our earlier comments on EP_3 receptor isoforms, this receptor may be an EP_3 receptor mediating overt contraction as opposed to the silent system found in the current study. Unfortunately, the activity of a recognized EP_3 receptor agonist was not reported by Zhang and colleagues.

Implications of prostanoid self-synergism in patho-physiological situations

Understanding the nature of the interaction between different prostanoid receptor systems is important, especially in the light of the recent intense interest in the clinical development of prostanoid receptor antagonists (see Jones *et al.*, 2009). In human platelets, EP_3 receptor agonists including PGE_2 markedly enhance aggregation due to a variety of agents (ADP, U-46619, PAF), while showing no response on their own (Mattthews and Jones, 1993). EP_3 receptor antagonists [relatives of (DG)-3ap] have been proposed for suppression of platelet activation associated with deterioration of atherosclerotic plaques (Heptinstall *et al.*, 2008). Activation of EP_3 and TP receptors mediating contraction of human blood vessels (see Jones *et al.*, 2009) may impinge on these events, but the nature of their (synergistic) interaction has received little attention. It seems unlikely that PGE_2 (or $PGF_{2\alpha}$) would be generated in sufficient concentration in patho-physiological situations to activate TP receptors and hence exert EP_3/TP self-synergism. The reverse situation whereby TXA_2 co-activates EP_3 and TP receptors is also unlikely. Notwithstanding, the EP_1 and EP_3 agonist potencies of PGE_2 are much closer. EP_1 receptors efficiently activate the Gq/PLC/Ca^{2+} release pathway (Funk *et al.*, 1993; Breyer *et al.*, 1996) and mediate strong contraction of smooth muscle. EP_1/EP_3 receptor self-synergism in blood vessels may be worthy of future investigation.

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Conflicts of interest

None.

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