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Characterization of a prostanoid EP_3-receptor in guinea-pig aorta: partial agonist action of the non-prostanoid ONO-AP-324

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1 Contraction of guinea-pig isolated aorta induced by the prostaglandin E analogue sulprostone (1–400 nM) has a lower maximum response (40%) than that of phenylephrine or U-46619 (TP-receptor agonist). A prostanoid EP_3-receptor subtype is involved based on agonist potency ranking: equi-effective molar ratios (EMR) are sulprostone ($EC_{50}\sim 23$ nM) 1.0, SC-46275 0.11, misoprostol 2.2, gemeprost 3.3, PGE_2 5.4, 17-phenyl PGE_2 6.0, GR-63799 8.9. GR-63799, which contains a bulky ester group, is relatively more potent on neuronal EP_3 preparations than on the aorta.

2 ONO-AP-324, a relative of the non-prostanoid prostacyclin mimetic series, behaves as an EP_3 partial agonist on the aorta, inhibiting sulprostone responses but acting synergistically (in a similar manner to sulprostone) with phenylephrine; it may be a useful pharmacological tool for studying EP_3-receptors.

3 Sulprostone contractions are markedly suppressed in zero-Ca^{2+} bathing fluid containing either 2 mM EDTA or 50 μM EGTA, and by Cd^{2+} (500 μM), but are usually unaffected by nifedipine (0.3 μM) and verapamil (4.44 μM). Influx of Ca^{2+}, but not through L-type Ca^{2+}-channels, appears to be the major contractile mechanism.

4 The guinea-pig aorta is a valuable addition to the vascular EP_3 preparations available and may increase our knowledge of the mechanisms whereby G_i-coupled receptors mediate vasoconstriction (c.f. 5-$HT_{1B/D}$- and α_2-receptors). The possibility of certain EP_3 agonists distinguishing EP_3-receptor isoforms is discussed.

Keywords: Arterial smooth muscle; prostanoid EP_3-receptors; prostaglandin E_2; sulprostone; non-prostanoid EP_3 agonists; non-prostanoid prostacyclin mimetics; L-type Ca^{2+}-channel blockers; G_i-coupled second messenger systems

Introduction

Prostaglandin E_2 (PGE_2) is generally found to be an arteriolar dilator through agonist actions at prostanoid EP_2- or EP_4-receptors. However, in a few vessels, for example rabbit renal artery (Ahluwalia *et al*., 1988) and human pulmonary artery (Qian *et al*., 1994), PGE_2 induces smooth muscle contraction at low concentrations. EP_3-receptors are involved and the PGE analogue sulprostone (Figure 1) serves as a useful EP_3 standard agonist due to its low EP_2/EP_4 potency.

The EP_3-receptor has received much attention of late due to the existence of several isoforms derived by alternative splicing of messenger RNA (Namba *et al*., 1993). The structural differences, which lie in the cytoplasmic tails, result in coupling to different second messenger systems. For example, when expressed in Chinese hamster ovary (CHO) cells, bovine adrenal gland EP_{3A} and EP_{3C} receptor isoforms couple to G_i to inhibit adenylate cyclase, EP_{3B} and EP_{3C} couple to G_s to activate adenylate cyclase, and EP_{3D}, as well as coupling to G_i and G_s, couples to Gq to cause pertussis toxin (PTX)-insensitive activation of phospholipase C (PLC) and Ca^{2+} mobilization (Namba *et al*., 1993). However, little is known about the ion channels/second messenger systems responsible for EP_3-receptor-mediated contraction of vascular smooth muscle.

We felt it important to find a suitable vascular EP_3 preparation for our intended Ca^{2+} flux and patch-clamp experiments. The human pulmonary artery was passed by due to its limited availability and the occurrence of tachyphylaxis to EP_3 agonists (Qian *et al*., 1994). The rabbit renal artery, briefly described by Ahluwalia *et al*. (1988), was indeed sensitive to sulprostone ($EC_{50}=10$ nM), but the maximum response was only 5–8% of the phenylephrine maximum (Jones, unpublished observations); it was reserved for later investigation. Of a number of other preparations, the guinea-pig aorta appeared to have the greatest potential. We shall deal with three aspects of its pharmacology. (a) The contractile potencies of a range of PGE analogues, indicating the presence of an EP_3-receptor. (b) The activity of ONO-AP-234 (Figure 1), a non-prostanoid found to be an EP_3 agonist (K. Kondo, ONO Pharmaceuticals, personal communication). ONO-AP-324 is chemically related to a series of non-prostanoid prostacyclin mimetics (Hamanaka *et al*., 1995a,b; Kondo & Hamanaka, 1995), one of which, ONO-1301 (Figure 1), we have also examined. (c) The effect of procedures that modify Ca^{2+} fluxes in smooth muscle cells on sulprostone contractions.

Methods

Isolated tissue preparations

Male Dunkin-Hartley guinea-pigs, weighing 400–450 g, were killed by cervical dislocation and exsanguination. The descending thoracic aorta was transferred to Krebs-Henseleit solution (NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.18, $NaHCO_3$ 25, glucose 10 mM). Following removal of adherent fat and connective tissue, 3 mm-wide rings were cut and suspended under 1.3 g tension in 10 ml organ baths

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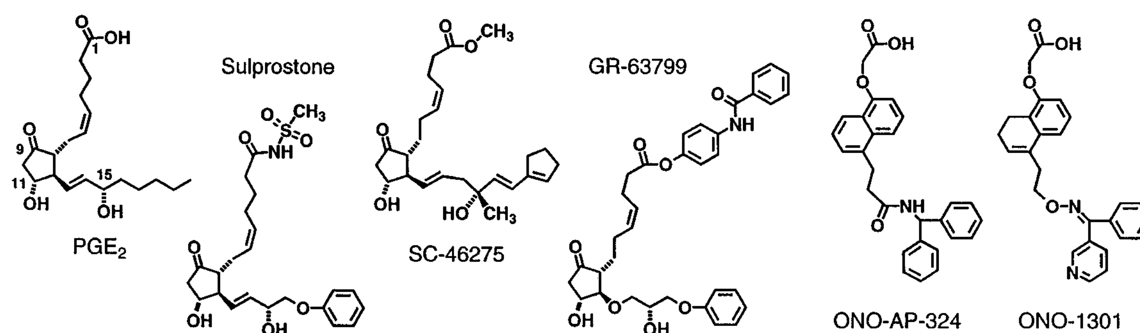

Figure 1 Chemical structures of PGE_2, its analogues sulprostone, SC-46275 and GR-63799, and the non-prostanoids ONO-AP-324 and ONO-1301. The -CONH- moiety in sulprostone is acidic due to the electron-withdrawing effect of the SO_2 group.

containing Krebs-Henseleit solution at 37°C and aerated with 95% O_2/5% CO_2. Isometric tension changes were recorded with Grass FT03 force transducers linked to a Maclab 4/ Macintosh PowerMac computer system (sampling rate 40 per min). The bathing solution contained 1 μM indomethacin in all experiments. In some experiments endothelium was removed by gentle rubbing with a wooden tooth-pick.

Ring preparations of human intralobular pulmonary arteries were obtained from lung lobes removed from patients with lung cancer at the Prince of Wales Hospital, Shatin. Experimental conditions were similar to those described for the guinea-pig aorta.

Contractions of the longitudinal smooth muscle of the guinea-pig isolated *vas deferens* to maximal electrical field stimulation (square wave pulses at 60 V, 1 ms, 10 Hz applied for 1 s) were recorded as described previously by us (Tam *et al*., 1997).

Experimental protocols

For vascular preparations, reproducible contractile responses to 40 mM KCl were first obtained on each preparation. A response to U-46619 (100 nM) was then obtained, against which all subsequent responses were normalized. Unless otherwise stated, all tests were performed in the presence of the TP antagonist GR-32191 (200 nM). For comparison of EP_3 contractile potencies, cumulative concentration-response relationships were obtained to two different PGE analogues on each preparation. For each analogue, half of the tests comprised the first cumulative sequence and half the second; PGE_2, misoprostol and gemeprost were not tested on the same preparation. The time between washout of the first agonist series and start of the second agonist series was 75–90 min. Although there appeared to be a slight increase in contractile sensitivity over time, applying 2-Factor ANOVA to $-\log EC_8$ values for the PGE analogues in Table 1 showed that the 'sequence' factor (i.e. whether the analogue was applied first or second) was not statistically significant ($P=0.18$); the PGE analogue factor had seven levels, excluding two sulprostone values secondary to gemeprost. For assessment of relaxant potencies, cumulative concentration-response relationships were obtained against tone induced by either 1 μM phenylephrine, 3 nM U-46619 or 300 nM sulprostone. To study the effects of receptor/ion channel blockers, matched preparations were exposed to either blocker or vehicle for 30 min and then cumulative dose-response relationships were obtained for the agonist.

On guinea-pig *vas deferens*, a single cumulative series of agonist doses was applied to each preparation.

Table 1 Contractile potencies of PGE analogues on guinea-pig isolated aorta

PGE analogue	*pEC_8 (−logM)*	*Equi-effective molar ratio*
SC-46275	9.41±0.04 (4)	0.11
Sulprostone	8.46±0.11 (8)	1.0
Misoprostol*	8.12±0.11 (4)	2.2
Gemeprost*	7.94±0.19 (4)	3.3
PGE_2*	7.73±0.06 (6)	5.4
17-Phenyl PGE_2	7.68±0.10 (4)	6.0
GR-63799	7.51±0.19 (4)	8.9

The TP antagonist GR-32191 (200 nM) was present in all tests. pEC_8 values are means±s.e.mean with *n* value in parentheses. *Lower maximum response than sulprostone due to opposing relaxant activity.

GraphPad Prism software was used for sigmoidal fitting of log concentration-response data, with constraint of the low response asymptote to 0% (contraction) or 100% (relaxation).

Compounds

The following compounds were gifts: sulprostone and cicaprost from Schering AG, Germany; misoprostol and SC-46275 (methyl 7-[2β-[6-(1-cyclopenten-1-yl)-4R-hydroxy-4-methyl-1E,5E-hexadienyl]-3α-hydroxy-5-oxo-1R,1α-cyclopentyl]-4Z-heptenoate) from GD Searle, U.S.A.; AH-6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid), GR-32191 (9α-(biphenylyl)methoxy-11β-hydroxy-12β-(N-piperidinyl)-ω-octanor-prost-4Z-enoic acid), GR-63799 (13-oxa-13,14-dihydro-16-phenoxy-ω-tetranor PGE_2 *p*-(benzoylamino)phenyl ester) from Glaxo Group Research, U.K.; butaprost from Bayer, U.K.; gemeprost (ONO-802) from ONO Pharmaceuticals, Japan: primary stocks were prepared in absolute ethanol at 2.5–10 mM. ONO-AP-324 (5-(2-diphenylmethylaminocarboxy)-ethyl)-α-naphthyloxyacetic acid) and ONO-1301 (7,8-dihydro-5-(2-(1-phenyl-1-pyrid-3-yl-methiminoxy)-ethyl)-α-naphthyloxyacetic acid) were gifts from ONO Pharmaceuticals, Japan: primary stocks in dimethylsulphoxide at 10 mM. U-46619, PGE_2, 17-phenyl-ω-trinor PGE_2 (17-phenyl PGE_2 in the text) and fluprostenol were purchased from Cayman Chemicals, U.S.A.: primary stocks in absolute ethanol at 5–10 mM. Nifedipine (10 mM in ethanol), cyclopiazonic acid (5 mM in dimethylsulphoxide), L-phenylephrine hydrochloride (10 mM in water), EDTA, EGTA and cadmium chloride were purchased from Sigma Chemicals, U.S.A. Verapamil hydrochloride was obtained from Knoll AG, Germany, as an aqueous injection solution

(2.5 mg ml^{-1}) for human use. All secondary stocks were prepared in 0.9% NaCl solution.

Results

Guinea-pig aorta

Sulprostone as the EP_3 standard agonist It was decided to use sulprostone as the standard EP_3-receptor agonist based on the following experiments, which were all carried out in the presence of 1 μM indomethacin to inhibit cyclo-oxygenase function.

The TP agonist U-46619 consistently contracted the aorta, with an EC_{50} of 9.6 ± 0.9 nM ($n=6$) and a maximum increase in tension of 2–3 g (Figure 2). For each preparation, tension changes were normalized to the 90–95% maximal response elicited by 100 nM U-46619. The TP antagonist GR-32191 (Lumley *et al.*, 1989) at concentrations of 5, 20 and 100 nM blocked the agonist action of U-46619 in a surmountable manner (Figure 2b). Regression analysis of a conventional Schild plot of the data yielded a slope of 1.06 (95% confidence limits = 0.76–1.46) and a pA_2 value of 9.15. This affinity is comparable with our previous pA_2 estimate of 9.43 for block of the TP-receptor in guinea-pig trachea by GR-32191 (Tymkewycz *et al.*, 1991). GR-32191 at 200 nM did not affect responses to sulprostone (1–400 nM, $n=4$), and was included in subsequent experiments.

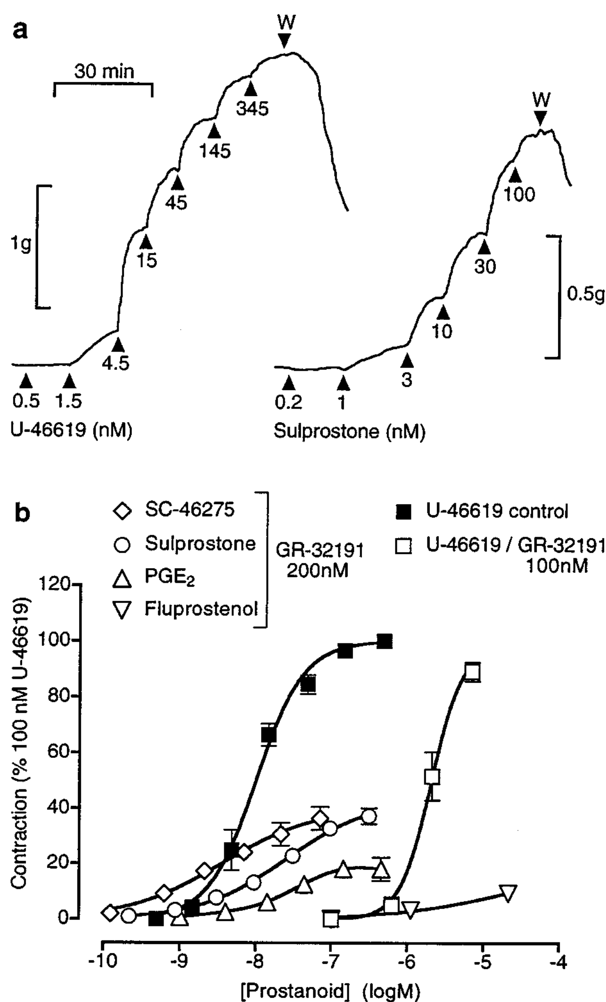

Figure 2 Contractile activity of prostanoids on the guinea-pig aorta ring preparation: (a) Typical tracing of responses to the TP agonist U-46619 (no receptor antagonists) and sulprostone in the presence of the TP antagonist GR-32191 (200 nM) on the same preparation. W = wash. (b) Log concentration-response curves for SC-46275, sulprostone, PGE_2 and fluprostenol in the presence of 200 nM GR-32191, and for U-46619 in the absence and presence of 100 nM GR-32191. Vertical bars show s.e.mean ($n=4-8$).

The contractures to sulprostone were stable, showed no reversal to relaxation at higher concentrations (Figure 2a), and no tachyphylaxis on repeated application over a 7 h period. The mean EC_{50} value for sulprostone was 23 nM and the maximum response some 40% of the U-46619 maximum (Figure 2b). Removal of the endothelium, which abolished the relaxant action of acetylcholine (1–14 μM) against phenylephrine-induced tone, had no effect on sulprostone responses ($n=3$); the endothelium was not removed in subsequent experiments. The EP_1 antagonist AH-6809 (Coleman *et al.*, 1985) at 5 μM did not block sulprostone contractions: dose ratio = 0.56 ± 0.03 ($n=3$) (data not shown). As a check of the genuineness of the AH-6809, it was tested at 2 μM against the contractile action of 17-phenyl PGE_2 on the guinea-pig isolated trachea (EP_1 preparation); a dose ratio of 63 ($n=1$) was obtained, with no change in responsiveness of a matching control preparation. The potent and selective FP-receptor agonist fluprostenol showed only weak contractile activity on the aorta (about 10% of the U-46619 maximum at 21.1 μM, Figure 2b).

Relative contractile potencies of PGE analogues Three PGE analogues, SC-46275 (Figures 1 and 2b), 17-phenyl PGE_2 and GR-63799, had log concentration-response curves apparently parallel to that of sulprostone. Three other analogues, PGE_2 (Figures 1 and 2b), misoprostol and gemeprost, had shallower curves with about 20% maxima, and were also distinguishable by the highest tested concentration (400–600 nM) inducing distinct relaxation in 4 of 6, 4 of 4, and 3 of 4 preparations respectively. Equi-effective molar ratios (sulprostone = 1.0, Table 1) were calculated from concentrations inducing 8% of the 100 nM U-46619 response; this response level is a compromise between the shallowness of the lower portion of the log concentration-response curve and the need to reduce interference from relaxant activity at higher agonist concentrations.

Gemeprost showed a much more persistent contraction following wash-out than did the other PGE analogues, with tension only returning to baseline after 3–5.5 h compared to about 1 h for sulprostone.

Relaxant actions of PGE analogues The final PGE analogue investigated was the selective EP_2 agonist butaprost. It did not contract the aorta when acting alone (10–1440 nM), but completely relaxed preparations precontracted (40–50%) with 1 μM phenylephrine ($IC_{50} = 163 \pm 20$ nM, $n=5$), 3 nM U-46619 ($IC_{50} = 42$ and 162 nM) or 300 nM sulprostone ($IC_{50} = 270$ and 660 nM). PGE_2, misoprostol and gemeprost (10–500 nM) each further contracted aorta preparations already contracted with 1 μM phenylephrine. However, somewhat variable relaxation responses were seen on preparations pre-contracted with 300 nM sulprostone: PGE_2 $IC_{50} = 98$, 390 and >440 nM; misoprostol $IC_{50} = 97$, 240 and >550 nM; gemeprost $IC_{50} = 190$, 380 and 420 nM.

Activity of the non-prostanoids ONO-AP-324 and ONO-1301 ONO-AP-324 (10 nM–14.4 μM) contracted the aorta, but its maximum response was only about 40% of that of sulprostone (Figure 3a). In the presence of 10 μM ONO-AP-

324, sulprostone responses were inhibited, whereas phenylephrine responses were enhanced (Figure 3b,c). A similar degree of synergism was also observed between sulprostone (3 nM) and either phenylephrine (0.1 – 10 μM) or U-46619 (1 – 100 nM, GR-32191 absent) (data not shown).

Two other non-prostanoids investigated, BMY-45778 (Seiler *et al.*, 1997) and ONO-1301, did not contract the aorta but did relax preparations contracted with 1 μM phenylephrine. Log concentration-relaxation curves for BMY-45778, ONO-1301 and the prostacyclin analogue cicaprost are shown in Figure 3d.

Effect of procedures that modify Ca^{2+} influx Initial experiments showed that reducing the external Ca^{2+} concentration with 2 mM EDTA in Ca^{2+}-free Krebs solution converted the sustained contraction to 10 μM phenylephrine to a large phasic response; higher EDTA concentrations also suppressed the phasic response. Responses to sulprostone (3 – 300 nM) in 2 mM EDTA/Ca^{2+}-free Krebs consisted of small tonic contractions (maximum response = 5 – 9% of 100 nM U-46619 response, $n = 3$). A similar profile for sulprostone was also seen in 50 μM EGTA/Ca^{2+}-free Krebs solution ($n = 2$), and the phenylephrine phasic response was prominent (see Low *et al.*, 1991). In both bathing fluids, subsequent addition of 2.5 mM Ca^{2+} rapidly restored both the tonic responses to phenylephrine and the much larger tonic response to sulprostone (35 – 48% of 100 nM U-46619 response). Treatment of the aorta with 500 μM Cd^{2+} for 30 min markedly inhibited sulprostone contractions, whereas U-46619 contractions were affected less (Figure 4).

The sarcoplasmic reticulum (SR) Ca^{2+}-pump inhibitor cyclopiazonic acid (10 μM) abolished the phasic response to 10 μM phenylephrine in 50 μM EGTA/Ca^{2+}-free Krebs solution. In normal Krebs solution 10 μM cyclopiazonic acid enhanced contractile responses to both U-46619 and sulprostone (Figure 4).

In preparations from more than 50 animals, sulprostone invariably showed a single exponential approach to a stable

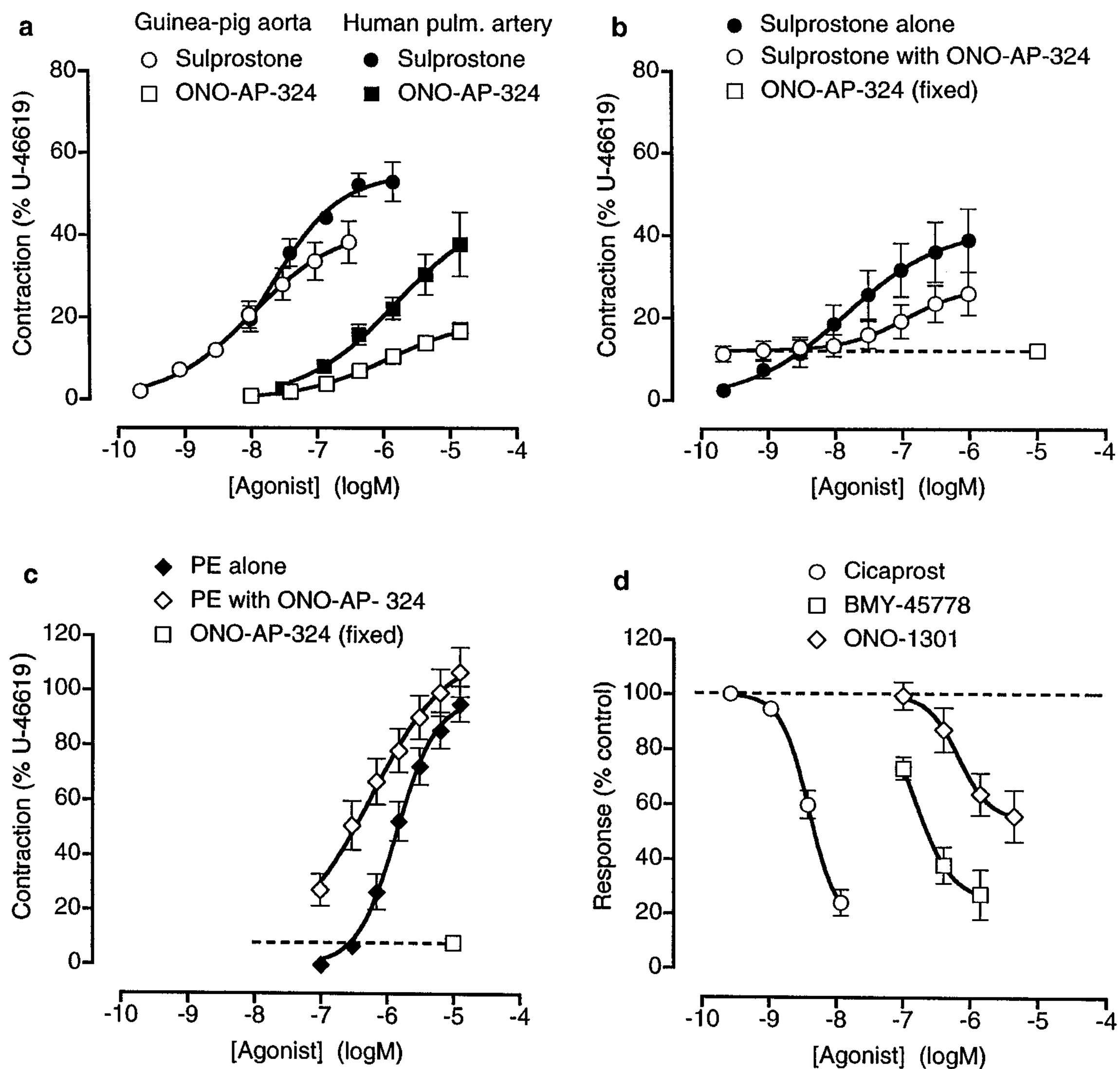

Figure 3 Vascular activity of the non-prostanoids ONO-AP-324, ONO-1301 and BMY-45778. Log concentration-response curves for (a) sulprostone and ONO-AP-324 both acting alone on guinea-pig aorta ($n = 6$) and human pulmonary artery ($n = 4$); (b) sulprostone acting alone and in the presence of 10 μM ONO-AP-324 on guinea-pig aorta ($n = 6$); (c) phenylephrine acting alone and in the presence of 10 μM ONO-AP-324 on guinea-pig aorta ($n = 6$); (d) inhibition of phenylephrine-induced tone by cicaprost ($n = 6$), ONO-1301 ($n = 3$) and BMY-45778 ($n = 3$) on guinea-pig aorta. The TP-receptor antagonist GR-32191 (200 nM) was present in all tests. Vertical bars show s.e.mean.

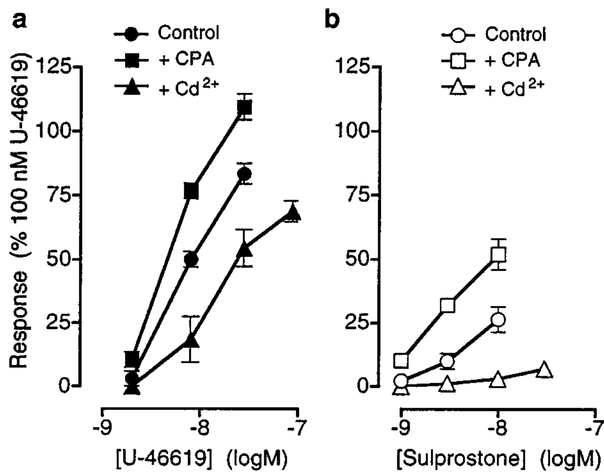

Figure 4 Guinea-pig aorta: effects of 10 μM cyclopiazonic acid (CPA) and 500 μM Cd^{2+} on log concentration-response curves to (a) U-46619 and (b) sulprostone. Vertical bars show s.e.mean ($n=6$ and 3 for control and treated preparations respectively).

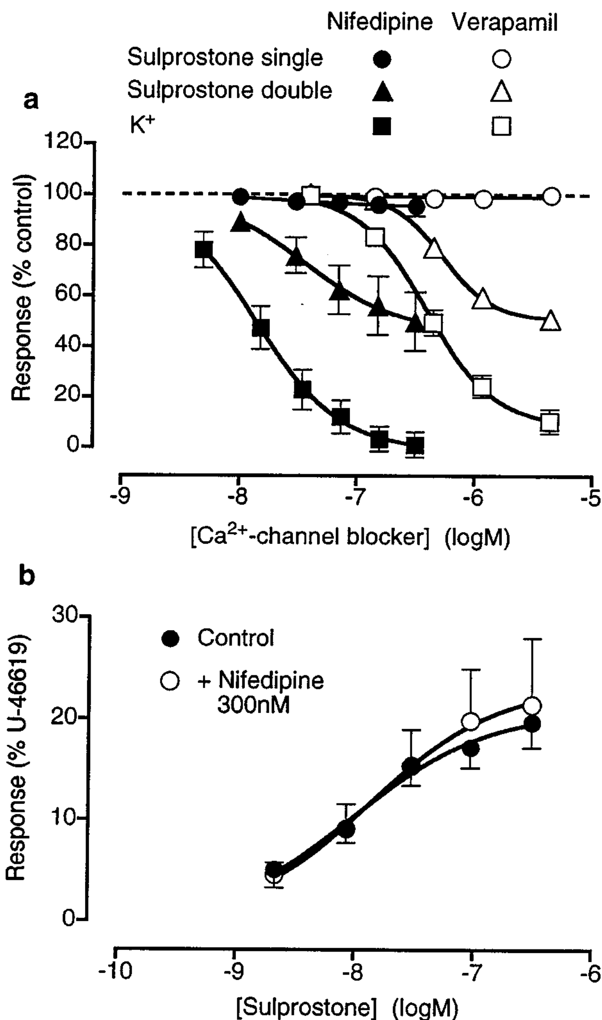

Figure 5 L-type Ca^{2+}-channel blockers on guinea-pig aorta: (a) Effect of nifedipine and verapamil on established contractions to 40 mM K^+ (both $n=6$), single stage contractions to 10 nM sulprostone ($n=5$ and 3 respectively), and double stage contractions to 10 nM sulprostone ($n=3$ and 1 respectively). (b) Effect of 300 nM nifedipine on the log concentration-response curve for sulprostone (both $n=4$). The TP-receptor antagonist GR-32191 (200 nM) was present in all tests. Vertical bars show s.e.mean.

contractile response; the L-type Ca^{2+}-channel blockers nifedipine (10–300 nM) and verapamil (40–4440 nM) did not inhibit these established responses in five and three preparations respectively (Figure 5a). Furthermore, in four of these typical experiments, pre-treatment of the preparation with 300 nM nifedipine for 30 min did not influence the sulprostone concentration-response curve (Figure 5b). However, in four other experiments performed early in the study sulprostone showed atypical contractions consisting of two tonic components of roughly equal size, with one commencing about 10 min after start of the first; in these cases addition of nifedipine (10–300 nM, $n=3$) and verapamil (40–4440 nM, $n=1$) produced a partial relaxation (Figure 5a). Nifedipine abolished and verapamil markedly inhibited contractile responses to 40 mM KCl ($IC_{50}=14$ and 450 nM respectively) (Figure 5a); nifedipine at 300 nM inhibited 1 μM phenylephrine contractions by 43–51% ($n=3$, data not shown).

Human pulmonary artery

In the presence of 200 nM GR-32191, ONO-AP-324 contracted the human pulmonary artery, with a threshold effect at 100 nM (Figure 3a). On preparations from two patients, log concentration-response curves to sulprostone and ONO-AP-324 were apparently parallel, whereas on preparations from two other patients the ONO-AP-324 curve was much shallower than that of sulprostone. The PGE analogue SC-46275, which was not examined in our previous study (Qian *et al.*, 1994) was found to be 4.3 ± 0.4 ($n=4$) times more potent than sulprostone as an EP_3 agonist on the pulmonary artery preparation.

Guinea-pig vas deferens

On the guinea-pig isolated *vas deferens*, sulprostone, PGE_2 and ONO-AP-324 completely inhibited maximal twitch responses elicited by electrical field stimulation. ONO-1301 showed only weak inhibitory activity, being about 140 fold less potent than ONO-AP-324 (Figure 6).

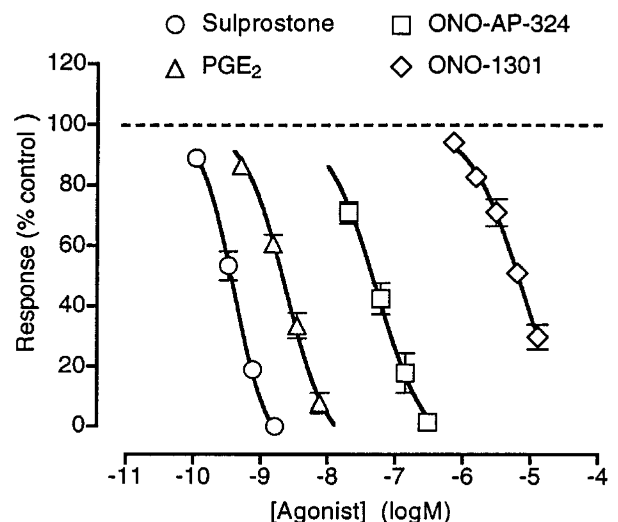

Figure 6 Inhibition of maximal twitch contractions of guinea-pig *vas deferens* elicited by electrical field stimulation: log concentration-responses curves for sulprostone, PGE_2 and the non-prostanoids ONO-AP-324 and ONO-1301. Vertical bars show s.e.mean ($n=4$ or 5).

Discussion

Characterizing the EP-receptor mediating vascular contraction

Several observations indicate that the contractile actions of PGE analogues on circular smooth muscle of the guinea-pig aorta are due to activation of EP_3-receptors. First, the most potent contractile agent SC-46275 has been shown to be a specific agonist for the EP_3-receptor (Savage *et al.*, 1993). Secondly, the potency ranking 'sulprostone > PGE_2 > 17-phenyl PGE_2' is characteristic of the EP_3-receptor, being reversed for the EP_1-receptor (Lawrence *et al.*, 1992). Thirdly, contractions elicited by sulprostone, which is a moderately potent EP_1 agonist (Coleman *et al.*, 1987a,b; Lawrence *et al.*, 1992), were not blocked by the EP_1 antagonist AH-6809. A contribution to PGE action from activation of TP-receptors is also unlikely, since PGE responses were observed in the presence of a concentration of GR-32191 giving a dose-ratio of about 300 for U-46619. Although none of the PGE analogues is noted for potent TP agonist activity, it was important to have a high degree of TP-receptor block in order to eliminate synergism between the EP_3 and TP contractile actions of a PGE analogue (as seen between sulprostone and U-46619). This situation is analogous to that in the rat aorta, where $PGF_{2\alpha}$ induces full contraction at micromolar concentrations. However, the presence of the TP antagonist SQ-29548 reveals a more sensitive but low-maximum ($\sim$10%) contractile component, which synergizes with noradrenaline and K^+ (Rapoport, 1993). Thus there is almost certainly synergism between the low potency TP agonism of $PGF_{2\alpha}$ and its more potent action through a second prostanoid receptor, probably an FP-receptor. On the guinea-pig aorta, the selective FP agonist fluprostenol (Welburn & Jones, 1978) was a very weak contractile agent, probably due to an agonist action at EP_3- rather than FP-receptors.

The relaxation seen with butaprost on the guinea-pig aorta over the 300–1000 nM concentration range has been reported previously for both EP_2 and EP_4 vascular preparations (Lawrence & Jones, 1992; Coleman *et al.*, 1994a; Lydford *et al.*, 1996), and its modest selectivity for EP_2-receptors is associated with EP_4 preparations having much higher sensitivity to PGE_2 than EP_2 preparations. Since it was not possible to measure the true relaxant potency of PGE_2 on the aorta, we shall not infer the subtype of EP-receptor involved. It is of interest that PGE_2 misoprostol and gemeprost each showed relaxant activity against sulprostone but additional contractile activity when phenylephrine was the tone-inducing agent. The explanation may be as follows. The tone induced by sulprostone (40% of the tissue maximum) should be ideal for PGE_2's relaxant action to show, whilst the EP_3 agonist action of PGE_2 should add little to that already present due to the near maximal concentration of sulprostone. With phenylephrine-induced tone, further contraction by PGE_2 should be favoured through synergism between the EP_3 and α_1 contractile systems and this could override relaxation.

Figure 7a shows that EP_3 contractile potencies on the guinea-pig aorta and the human pulmonary artery are well correlated. Indeed, the prostanoid receptor complements of the two preparations are similar, with each containing contractile EP_3- and TP-receptors and relaxant EP- and IP-receptors. Turning to within-species comparisons and the possible relevance of EP_3-receptor isoforms to our study, EP_3 agonist potencies on the guinea-pig aorta and the guinea-pig *vas deferens* (Lawrence *et al.*, 1992) are not so well correlated, particularly for 17-phenyl PGE_2 and GR-63799 (Figure 7b). Data on the guinea-pig trachea obtained by another research group (Spicuzza *et al.*, 1998) also shows a higher EP_3 potency for GR-63799 compared to PGE_2 and 17-phenyl PGE_2 (Figure 7b). The EP_3-receptors in the *vas deferens* and the trachea are both located presynaptically and suppress transmitter release from sympathetic and parasympathetic nerve terminals respectively. GR-63799 (Bunce *et al.*, 1990) is different to the other PGE analogues examined in containing a relatively bulky *p*-(benzoylamino)phenyl ester (Figure 1). It is possible that GR-63799 could activate EP_3-receptors as either the native ester or the (de-esterified) carboxylic acid. Two scenarios may be envisaged. Based on the results of conventional metabolism studies *in vivo*, it is generally

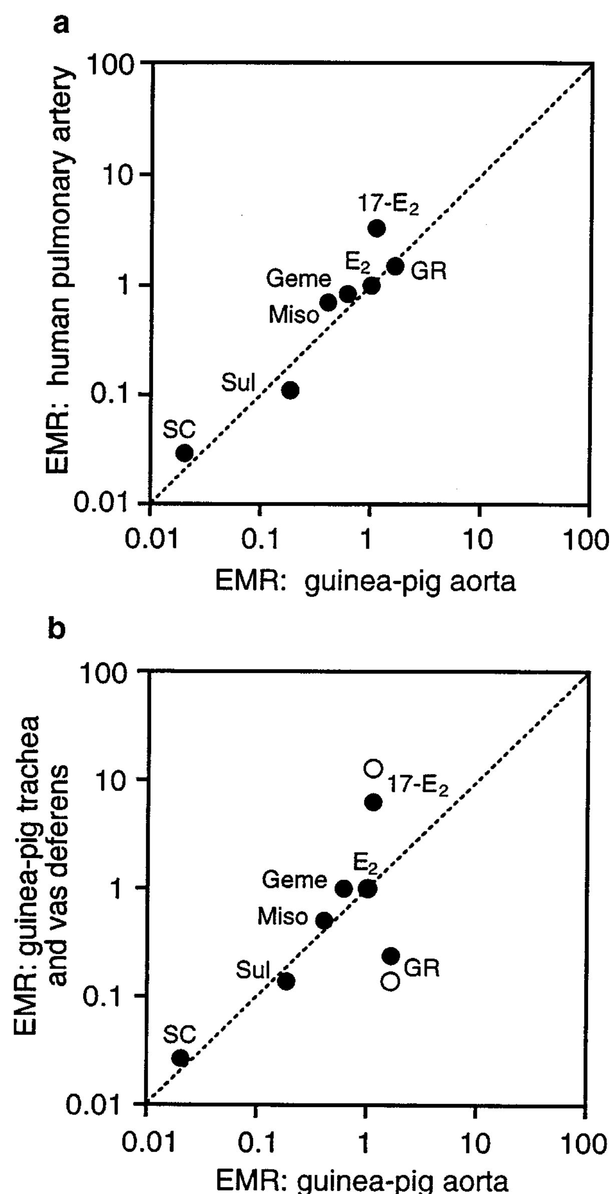

Figure 7 Comparison of EP_3 agonist potencies: (a) guinea-pig aorta *versus* human pulmonary artery and (b) guinea-pig aorta *versus* guinea-pig *vas deferens* (●) and guinea-pig trachea (○). Data taken from this study, Lawrence *et al.* (1992), Qian *et al.* (1994) and Spicuzza *et al.* (1998). PGE_2 is the standard agonist in each case (equi-potent molar ratio, EMR = 1.0). Compound codes: SC = SC-46275, Sul = sulprostone, Miso = misoprostol, Geme = gemeprost, E_2 = PGE_2, GR = GR-63799, 17-E_2 = 17-phenyl PGE_2. Each diagonal line represents absolute equivalence between EMRs, not a linear regression for the data points.

supposed that simple alkyl ester prostanoids (e.g. SC-46275 and misoprostol) are rapidly hydrolyzed in isolated tissues to afford the more biologically-active free acids. However, it is not clear that the more bulky *p*-(benzoylamino)phenyl ester in GR-63799 would be rapidly hydrolyzed; if the rates were to differ in different tissues, then relative potencies would be affected. More interestingly, if native GR-63799 is the active species and the vascular and neuronal EP_3-receptors are different isoforms operating through different G-proteins, then the binding of the extended α-chain of GR-63799 within the transmembrane region may produce a selective disturbance of G-protein coupling. Although cytoplasmic tail splicing in EP_3 isoforms affects both the type of G-protein coupling, the rate of agonist desensitization and the degree of constitutive activity (Negishi *et al*., 1989; An *et al*., 1994; Jin *et al*., 1997), major effects on relative agonist affinities have not been apparent. For example, PGE_2, PGE_1, $PGF_{2\alpha}$ and M&B-28767 have similar binding affinities for the isoforms of the human EP_3-receptor expressed in CHO and COS-7 cells (An *et al*., 1994; Regan *et al*., 1994). However, a large group of EP_3 agonists of widely differing structure has not been investigated, and it would be of interest to pursue this line of enquiry by examining PGE analogues with radically different substituents on the ester (or sulphonamide) unit to see if larger differences in EP_3 agonist potency are to be found.

EP_3 agonist activity of the non-prostanoid ONO-AP-324

ONO-1301 (Kondo & Hamanaka, 1995) is a member of a group of non-prostanoids that show IP agonist activity on both platelets and arterial smooth muscle (Hamanaka *et al*., 1995a,b). Activity is optimal when the 1-carboxylate is situated at a critical distance from the diaryl moiety, the latter being crucial to the IP agonism (see also Jones *et al*., 1993; Meanwell *et al*., 1994). ONO-1301 also inhibits thromboxane synthetase (Kondo & Hamanaka, 1995), but this will be of no consequence in our experiments since endogenous synthesis of PGH_2 was inhibited by the cyclo-oxygenase inhibitor indomethacin. The closely related analogue ONO-AP-324 is unique in that it behaves as a selective EP_3 agonist, competing with [^{3}H]PGE_2 for the mouse cloned $EP_{3\alpha}$-receptor expressed in CHO cells with a K_i of 11 nM. Its specificity is high, with K_i values for mouse EP_2-, EP_4- and FP-receptors and human IP- and TP-receptors being greater than 10 μM, and for mouse EP_1-receptors being 4.6 μM (K. Kondo, personal communication). In our functional study, ONO-AP-324 also appeared to show EP_3 agonist activity on the guinea-pig aorta, but its maximum response was less than that of sulprostone and it opposed the contractile action of sulprostone. Two ready explanations for this profile must be considered: either ONO-AP-324 has IP agonist activity that opposes it (full) agonist activity at the contractile EP_3-receptor or it is a partial agonist at the EP_3-receptor. Although ONO-AP-324 does not bind to the human cloned IP-receptor, we must be cautious in arguing from this information, since there are species differences in the sensitivity of IP-receptor systems to the diaryl non-prostanoids, with human, pig and horse being more sensitive than rat and rabbit (Armstrong *et al*., 1989). The guinea-pig IP-receptor falls into the insensitive group, with octimibate having much weaker inhibitory activity (relative to iloprost, EMR = 1.0) on guinea-pig washed platelets (IC_{50} = 204 nM, EMR = 296) compared to human washed platelets (IC_{50} = 10.5 nM, EMR = 12) (Merritt *et al*., 1991). Also, BMY-45778, one of the most potent non-prostanoid prostacyclin mimetics on human platelets (Meanwell *et al*., 1994; Seiler *et al*., 1997), has an IC_{50} of about 3 nM for relaxation of human pulmonary artery (Jones *et al*., 1997) compared to 240 nM on the guinea-pig aorta (this study). And in addition, ONO-1301 competes for [^{3}H]iloprost binding on human platelets with a K_i of 190 nM (Kondo & Hamanaka, 1995), whereas it showed only weak relaxant activity on guinea-pig aorta (IC_{50} > 5 μM). Thus there is no reason to suppose that ONO-AP-324 is acting as an IP agonist on the guinea-pig aorta. Supporting this, and favouring the partial agonist proposal, is our observation that the synergism between ONO-AP-324 and phenylephrine is of a similar magnitude to that between sulprostone and phenylephrine.

On the field-stimulated guinea-pig *vas deferens*, ONO-AP-324 behaved as a full EP_3 agonist. There is not necessarily a conflict with the guinea-pig aorta findings, since the *vas deferens* is much more sensitive to EP_3 agonists (sulprostone IC_{50} = 0.36 nM) compared to the aorta (sulprostone EC_{50} = 23 nM) and it is often the case that partial agonists behave as full agonists on more sensitive preparations (Kenakin *et al*., 1992).

Partial agonist activity at EP-receptors among the many PGE analogues synthesized seems to be rare, making this avenue to EP-receptor antagonists unattractive. The apparent partial agonist action of ONO-AP-324 on the guinea-pig aorta EP_3 system is therefore of great interest. The relatively simple structure of ONO-AP-324, coupled with the absence of chirality, might encourage approaches to an EP_3 antagonist based on combinatorial chemistry and large-scale ligand-binding screens.

Mechanism of the vascular contraction induced by EP_3 agonists

The activation of receptors (TP, α_1, AT_1) that interact with PLCβ *via* G_q usually leads to full contraction of isolated arterial and venous smooth muscle preparations. This is not surprising since several contractile systems are set in motion (see Kuriyama *et al*., 1995). Inositol 1,4,5-trisphosphate ($InsP_3$) releases Ca^{2+} from the SR and diacylglycerol activates protein kinase C to enhance sensitivity of the contractile elements to Ca^{2+}; Ca^{2+} influx through opening of L-type and other Ca^{2+}-channels in the plasma membrane is also involved (see Coleman *et al*., 1994b for information on TP-receptors). Recently, a Rho-associated protein kinase has been implicated in Ca^{2+}-sensitization in pig coronary artery and guinea-pig trachea (Uehata *et al*., 1997). In the case of EP_3 agonist action on the guinea-pig aorta, the marked suppressant effects of both extracellular Ca^{2+} depletion and Cd^{2+} (a non-specific Ca^{2+}-channel blocker), the insensitivity to L-type Ca^{2+}-channel blockers, and the lower maximum response suggest a different contractile mechanism. One possibility, by analogy with studies of ATP, noradrenaline and acetylcholine action on isolated vascular smooth muscle cells (review by Kuriyama *et al*., 1995), would involve influx of Ca^{2+} through receptor-operated channels, which may be selective for Ca^{2+} (Murray & Kotlikoff, 1991) or non-selective for cations (Benham & Tsien, 1987). Patch clamp experiments on isolated smooth muscle cells are in progress to investigate this situation.

In Ca^{2+}-free bathing fluid, the SR Ca^{2+}-pump inhibitor cyclopiazonic acid (see Darby *et al*., 1996 for selectivity data) inhibited tonic contractions of the guinea-pig aorta to phenylephrine, as found in other vascular preparations (Deng & Kwan, 1991). The potentiation of sulprostone and U-46619 contractions by cyclopiazonic acid in the presence of a normal level of external Ca^{2+} is similar to that seen on 5-HT and K^+ contractions of rat small mesenteric artery (Shima & Blaustein, 1992) and can be explained by suppression of the buffering effect of Ca^{2+} uptake into the SR, thus allowing Ca^{2+} influx to

more effectively activate the contractile machinery (Shima & Blaustein, 1992). In a few guinea-pig aorta preparations studied early in the project, sulprostone elicited a secondary nifedipine-sensitive tonic contraction, presumably due to membrane depolarization reaching a level sufficient to open L-type Ca^{2+}-channels. We have not observed this phenomenon in later experiments and the reason for this is not clear.

The actions of EP_3 agonists on platelet, renal, and gastric function have been associated with receptor coupling to G_i and subsequent inhibition of adenylate cyclase by the α subunit (see Coleman *et al*., 1994b). A similar mechanism may underlie EP_3 vascular contraction and also synergy with phenylephrine. Sumatriptan, a selective $5\text{-}HT_{1B/D}$ agonist, contracts dog saphenous vein and reduces both basal and PGE_2-stimulated cyclic AMP levels over similar concentration ranges (Sumner & Humphrey, 1990). However, obtaining good evidence for contraction through adenylate cyclase inhibition can be difficult, since the requisite ongoing production of cyclic AMP must suppress a contractile influence, the nature and strength of which may be difficult to determine.

In many vessels, activation of $5\text{-}HT_1$- (MacLennan *et al*., 1993; Sweeney *et al*., 1995), muscarinic- (Asano & Hidako, 1980) and NPY_1-receptors (Edvinsson *et al*., 1984), all of which potentially couple to G_i, typically results in minimal vasoconstriction and marked enhancement of the contractile activity of a second agonist acting through a conventional receptor/G_q/PLCβ system. A mechanism which readily accommodates this profile involves the $\beta\gamma$-subunit of G_i augmenting $G_q\alpha$-activated PLCβ activity (Katz *et al*., 1992; Camps *et al*., 1992; Smrcka & Sternweis, 1993; Wu *et al*., 1993; Selbie *et al*., 1997; see review by Selbie & Hill, 1998). Due to the existence of different PLCβ isoforms and different $\beta\gamma$ subunits (e.g. $\beta_1\gamma_1$, $\beta_1\gamma_2$), considerable variation in $InsP_3$ and diacylglycerol levels in the cell is possible, leading to different agonist profiles (Wu *et al*., 1993). In the case of EP_3 vascular contraction, a first step would be to measure the effects of sulprostone alone and in combination with phenylephrine or U-46619 on PLC activity in isolated vessels or dispersed smooth muscle cells.

How do our observations fit in with what is known about agonist mechanisms of cloned EP_3-receptors transfected into the usual cell lines? Coupling to adenylate cyclase through G_i is a consistent phenomenon, whereas elevation of internal Ca^{2+} shows variable properties. For example, in the mouse, there are three EP_3 isoforms, α, β and γ that couple to G_i to inhibit adenylate cyclase, ($EP_{3\gamma}$ also couples to G_s to raise cyclic AMP levels at higher agonist concentrations) (Irie *et al*., 1993). PGE_2 activates all three mouse EP_3-receptor isoforms expressed in CHO cells to raise $InsP_3$ and Ca^{2+} levels transiently, and these effects are inhibited by both PTX and the PLC inhibitor U-73122 (Irie *et al*., 1994). Between 60 and 80% of the Ca^{2+} signal is due to influx from the extracellular space. Thus, the EP_3-receptor couples to G_i to activate PLC, leading to Ca^{2+} mobilization from internal stores and influx from the extracellular space. In contrast to the mouse data, the rise in Ca^{2+} level induced by M&B-28767 in CHO cells expressing human EP_3 isoforms was not effected by removal of extracellular Ca^{2+} and only slightly depressed by PTX (An *et al*., 1994), and the elevation of $InsP_3$ by PGE_1 and PGE_2 in CHO cells expressing a bovine EP_3-receptor was completely insensitive to PTX (Negishi *et al*., 1989).

In summary, our work on the contractile action of EP_3 agonists on guinea-pig aorta points to the need to establish the mechanism(s) involved, which may be novel. In addition, greater knowledge of the cardiovascular actions of EP_3 agonists, either in current therapeutic use (e.g. misoprostol) or of potential use (e.g. non-prostanoid mimetics), could be of considerable value. Indeed, sulprostone has recently been shown to inhibit myocardial infarct size in the rabbit (Hide & Thiemermann, 1996).

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