Pharmacological characterization *in vitro* of prostanoid receptors in the myometrium of nonpregnant ewes

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Prostanoid receptors regulating the contractility of strips of myometrium obtained from nonpregnant ewes during the breeding season were classified pharmacologically. Natural prostanoids, receptor-type selective synthetic analogues, and selective antagonists were used where available. The natural prostanoids PGD₂, PGE₂, and PGF_{2α} were equipotent in causing contractions (pD_2 values of 6.9, 6.7, and 6.9, respectively) but were 100 times less potent than oxytocin ($pD_2 = 9.2$). The synthetic prostanoids iloprost ($pD_2 = 8.3$), GR63799x ($pD_2 = 7.0$), cloprostenol ($pD_2 = 6.8$), and U46619 ($pD_2 = 6.2$) also caused contractions. The effects of iloprost, but not of GR63799x, were blocked by the selective EP₁ antagonist AH6809. This suggests the presence of both EP₁ and EP₃ receptors. The similar potencies of cloprostenol and PGF_{2α} suggest the presence of FP receptors. Although the potency of the TP agonist U46619 was relatively low, its effects were blocked by the selective TP antagonist L670596 ($pK_B = 8.4$), an observation consistent with the presence of TP receptors. Thus, all currently recognized excitatory prostanoid receptors (EP₁, EP₃, FP and TP) appeared to be present. Contractions induced by cloprostenol and KCl could be inhibited by the β -adrenoceptor agonist isoprenaline ($pD_2 = 8.8$ against cloprostenol) and the Ca²⁺-channel blocker, D600 ($pD_2 = 6.3$ against cloprostenol), but a number of relaxant prostanoids, BW245c, ZK110841, AH13205 and cicaprost, could not produce inhibition. These results suggest that DP, EP₂ and IP receptors do not regulate contractility under these conditions.

Introduction

Novy and Liggins (1980) suggest that prostanoids play a physiological role in the regulation of uterine activity in a number of species. In ewes, work aimed at delineating this role has focused, almost exclusively, on alterations in prostanoid concentrations in various compartments during different phases of the reproductive cycle (Olson *et al.*, 1986). Scant attention has been paid to the actions of these compounds at the putative end-organ.

In humans (Senior *et al.*, 1991, 1992, 1993), the direct effects of prostanoids on uterine contractility are mediated through actions at seven different receptors, some of which are excitatory, others inhibitory. In ewes, however, the prostanoid receptor status of the myometrium is not known.

In the present study, the prostanoid receptor status of the myometrium of nonpregnant ewes during the breeding season was determined pharmacologically by studying the effects of prostanoid receptor-selective analogues on the contractility of the myometrium *in vitro*.

Materials and Methods

Collection of myometrium

Reproductive tracts were obtained from two abattoirs in the vicinity of Hamilton, Ontario (43°15'N). They were collected

between 15 September and 15 December over three successive seasons, from crossbred yearling ewes or ewe lambs weighing more than 40 kg. The tracts were placed in ice cold physiological salt solution (PSS) of the following composition (mmol l⁻¹): potassium chloride 4.6, magnesium sulfate 1.16, sodium dihydrogen phosphate 1.16, calcium chloride 2.5, sodium chloride 115.5, sodium bicarbonate 21.9 and glucose 11.1 with indomethacin at 10 μ mol l⁻¹, and transported to the laboratory. In the laboratory, the ovaries were inspected for indications of reproductive status. Only uteri from animals with an active corpus luteum, or large fluid-filled follicles accompanied by a recently regressed corpus luteum in at least one ovary, were included in the study. Results from uteri from 45 animals that met these criteria are reported. None of these reproductive tracts showed any indication of trophoblastic activation. The uteri were washed with oxygenated (95% O₂:5% CO₂) PSS at room temperature, cleared of any adhering fat and connective tissue, and eight longitudinal strips of myometrium approximately 1.5 cm in length and 0.04 cm² in cross-sectional area were cut from the body of the uterus.

Recording of isometric contractions

General protocol. Tissue strips were tied at each end with silk thread and mounted longitudinally in individual 10 ml jacketed muscle baths containing oxygenated PSS at 37°C. One end of each strip was anchored in the bath; the other was

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Table 1. Composition, concentration and sources of compounds used

Compound	Stock solution	Source
17-phenyl trinor PGE ₂	10 ⁻³ g ml ⁻¹ in absolute ethanol	Cayman Chemical, Ann Arbor, MI
AH6809	2.5 × 10 ⁻³ g ml ⁻¹ in 1% (w/v) NaHCO ₃	Glaxo Group Research, Ware
AH13205	10 ⁻² g ml ⁻¹ in 1% (w/v) NaHCO ₃	Glaxo Group Research
BW245c	10 ⁻² mol l ⁻¹ in absolute ethanol	Wellcome Research Laboratories, Beckenham
BW A868c	10 ⁻² mol l ⁻¹ in absolute ethanol	Wellcome Research Laboratories
Carbachol	10 ⁻¹ mol l ⁻¹ in 0.9% (w/v) saline	Sigma Chemical Co., St Louis, MO
Cloprostenol	10 ⁻³ g ml ⁻¹ in 0.9% (w/v) saline	ICI Pharmaceuticals, Alderley Park
Cicaprost	5 × 10 ⁻⁵ g ml ⁻¹ in aqueous solution	Schering AG, Berlin
D600	10 ⁻³ mol l ⁻¹ in 0.9% (w/v) saline	Sigma Chemical Co.
GR63799x	10 ⁻² g ml ⁻¹ in absolute ethanol	Glaxo Group Research
Iloprost	10 ⁻⁵ g ml ⁻¹ in aqueous solution	Schering AG
Indomethacin	5 × 10 ⁻³ mol l ⁻¹ Na ₂ CO ₃	Sigma
Isoprenaline	10 ⁻² mol l ⁻¹ in 0.9% (w/v) saline and ascorbate	Sigma
L670596	10 ⁻³ g ml ⁻¹ in DMSO	Merck Frosst Centre for Therapeutic Research, Pointe Clare, PQ
Misoprostol	10 ⁻² g ml ⁻¹ in absolute ethanol	G. D. Searle, Skokie, IL
Oxytocin	10 ⁻³ mol l ⁻¹ in 0.9% (w/v) saline	Vega, Tucson, AZ
PGD ₂	10 ⁻² mol l ⁻¹ in absolute ethanol	Cayman Chemical
PGE ₂	10 ⁻² mol l ⁻¹ in absolute ethanol	Cayman Chemical
PGF _{2α} tromethamine salt	10 ⁻² mol l ⁻¹ in 0.9% (w/v) saline	Cayman Chemical
Sulprostone	10 ⁻³ g ml ⁻¹ in aqueous solution	Schering AG
U46619	10 ⁻² g ml ⁻¹ in methyl acetate	Cayman Chemical
ZK110841	1.25 × 10 ⁻³ g ml ⁻¹ in absolute ethanol	Schering AG

attached to an FT-03 force displacement transducer writing to a 7D polygraph (Grass Instruments, Quincy, MA). An initial resting force of 40 mN was applied to each tissue. This usually declined rapidly and was readjusted throughout the next hour to obtain a steady baseline force of 25 mN. Throughout this period, the bath solution was changed regularly. Tissues were then challenged with potassium chloride (90 mmol l⁻¹). Any strip that failed to develop force in response to this challenge was discarded. Potassium chloride was washed from the bath and tissues were allowed to equilibrate for a further hour. The mean force developed by the individual muscle strips during 10 min was used as a measure of their contractility. This technique has been used routinely in this laboratory (Dyal and Crankshaw, 1988; Wainman *et al.*, 1988) and others (Cheuk *et al.*, 1993), since it can be used to quantify drug effects in tissues that develop significant spontaneous activity and respond to stimulation by changes in both tonic and phasic activity (Crankshaw, 1990). Mean force was determined exactly as described by Wainman *et al.* (1988).

Concentration–effect curves to excitatory agonists

At the end of the equilibration period, the mean force developed during a 10 min control period was determined. Drugs were then added to the baths, in a cumulative fashion, by increments that would produce approximately one half log unit changes in the concentration in the bath. After each addition, the mean contractile force was determined for 10 min. The mean force recorded in 10 min immediately after agonist addition minus the mean control force was considered to be the

force developed in response to that concentration of agonist. Additions were continued until further addition produced no further increase in developed force. Concentration–effect curves (response versus log molar agonist concentration) were then constructed from these data by fitting the equation

$$E = E_{min} + (E_{max} - E_{min}) / (1 + e^{-k(\log C - \log D)})$$

where E is the effect of the agonist, C is the molar concentration of the agonist and D is the molar concentration of the agonist that produces a half maximal response (EC_{50}). The value of $-\log D$ is equivalent to the negative $\log_{10}$ of the molar concentration of the agonist that produces 50% of the maximal response, pD_2 values.

Normally the same agonist was tested on two muscle strips from the same animal and the average of the two pD_2 values obtained was used. Thus n values represent the number of animals on which each drug was tested.

Concentration–effect curves to inhibitory agonists

Although most tissues developed spontaneous contractile activity during the equilibration period, this activity decayed with a time course that was highly variable, particularly between animals, but also between strips from the same animal. This made it difficult to obtain concentration–effect data for inhibitors. To remedy this problem, strips were stimulated in two different ways, and the effect of drugs on this stimulated activity was examined. At the end of the equilibration period, the PSS was changed to a solution that contained either 40 mmol potassium chloride l⁻¹ or 2 μmol cloprostenol l⁻¹.

Fig. 1. The effect of the EP receptor agonist, GR63799x, on the contractility of the sheep myometrium *in vitro*. Record of force development during the experiment showing the manual setting of initial tensions and immediate development of high frequency, low amplitude contractions (A), the development of low frequency, high amplitude contractions (B), a contraction induced by KCl (C) and the concentration effect in the experiment (D).

Strips were left for 1 h before concentration–effect experiments were begun. Procedures for these experiments were similar to those for excitatory agonists, except that two strips out of every eight were used as time-matched controls, and the response was a decrease in contractile activity. At the end of the experiment, the L-type calcium channel blocker D600 was added to each tissue bath at a final concentration of $100 \mu\text{mol l}^{-1}$. After exposure to D600 for 15 min, a final data collection was performed. We expected D600 to reduce activity to a basal tone and therefore used this maximal D600 response to define zero contractility. The zero contractility value was subtracted from all preceding values, which were then expressed as a percentage of the control values. Drug effects were then calculated according to the following equation:

$$E_x = ((D_x - C_x) / -C_x) \times 100$$

where E_x is the percentage inhibition produced by the drug at concentration x , D_x is the percentage of control period activity developed in the presence of concentration x of the drug, and C_x is the percentage of control period activity developed in the time-matched control tissues for the same period.

Effects of antagonists

In attempts to delineate the site of action of a number of excitatory agonists, experiments were performed to investigate the effects of selective receptor antagonists on their action. The general protocol as described above was used, except that four tissue strips from each animal were used. Two tissues were incubated in the presence and two in the absence of the specified antagonist for 1 h before control data were obtained, and throughout the course of the concentration–effect experiment. The pD_2 values obtained in the presence of antagonist were compared with those obtained in its absence. Where appropriate, pK_B (negative $\log_{10}$ of the molar concentration of antagonist that would occupy 50% of the receptors in the absence of agonist) values for the antagonists were calculated using the Gaddum equation:

$$pK_B = \log (\text{concentration ratio} - 1) - \log (\text{antagonist concentration})$$

Where concentration ratio = EC_{50} value in the presence of antagonist: EC_{50} value in the absence of antagonist.

Drugs

The compounds used in this study, the composition and concentration of stock solutions, and the source of the compounds are all listed in Table 1. Dilutions from stock solutions were made on the day of the experiment directly into saline and were kept on ice for the duration of the experiment. Stock solutions were maintained as recommended by the suppliers, except for carbachol, isoprenaline and sulprostone which were made up freshly for each experiment, and indomethacin which was treated according to the method of Curry *et al.* (1982). The aqueous solutions for iloprost and cicaprost were as supplied by the manufacturer. All other chemicals were from BDH (Toronto).

Fig. 2. Detail of Fig. 1 showing the concentration effect experiment. Horizontal bars indicate data collection for 10 min, the first of which is the control. Thereafter, the EP receptor agonist, GR63799x, was added cumulatively at the arrows to give concentrations in the bath ranging from 1 nmol l^{-1} to $1.4 \mu\text{mol l}^{-1}$.

Fig. 3. Concentration-effect curve to the EP receptor agonist GR63299x resulting from the data in Fig. 2. The last two collections in this experiment ($\blacktriangle$) produced activities that were lower than the maximum, and were not therefore included in the determination of the pD_2 value.

Results

When incubated under the conditions described, 80% of the myometrial strips used developed high frequency (1–5 min⁻¹), low amplitude (about 10 mN) spontaneous contractions. The remainder were quiescent. Lack of development of spontaneous contractility was not correlated with failure to respond to potassium chloride challenge or to prostanoid addition. Of those tissues that developed spontaneous contractions, 15% also developed low frequency (0.3–0.5 h⁻¹), high amplitude (about 20 mN) contractions.

Concentration-dependent increases in the contractile activity of the sheep myometrium *in vitro* were produced by the prostanoids PGD₂, PGE₂, PGF_{2α}, iloprost, 17-phenyltrnor

PGE₂, sulprostone, misoprostol, GR63799x, cloprostenol and U46619, and also by oxytocin and carbachol. Conversely, the prostanoids BW245c, ZK110841, AH13205, and cicaprost were not capable of increasing contractile activity at concentrations up to 30, 10, 80 and 0.05 μmol l⁻¹, respectively.

Figure 1 shows the complete tension recording from one of the experiments investigating the effects of an excitatory agonist (GR63799x). The concentration-effect portion of this experiment is shown in Fig. 2 and the resulting concentration-effect curve in Fig. 3. Table 2 shows the potencies of all the agonists tested that had excitatory effects.

There were no differences in the pD_2 values obtained for 17-phenyltrnor PGE₂ and GR63799x in the presence or in the absence of the EP₁ antagonist AH6809 (Coleman *et al.*, 1985) at 40 μmol l⁻¹ ($n = 4$). Conversely, the concentration-effect curves for iloprost were displaced so far to the right in the presence of AH6809 that reliable pD_2 values could not be determined with the concentrations of the agonist available.

The selective DP receptor antagonist BW A868c (Giles *et al.*, 1989) at 1 μmol l⁻¹ had no effect on responses to PGD₂. The selective TP receptor antagonist, L670596 (Ford-Hutchinson *et al.*, 1989) shifted the concentration-effect curves for U46619 to the right. Control pD_2 values were 6.34 ± 0.29 ; those in the presence of L670596 were 5.79 ± 0.2 , giving a mean pK_B for L670596 of 8.4.

The β-adrenoceptor agonist isoprenaline and the Ca-channel antagonist D600 caused concentration-dependent inhibition of KCl- and cloprostenol-induced activity. Figure 4a shows the decline in cloprostenol-induced activity with time and Fig. 4b shows the decline in cloprostenol-induced activity in a matched strip that was treated with isoprenaline. The response to isoprenaline was biphasic, showing excitation at very high concentrations, whereas the response to D600 (not shown) was monophasic. The pD_2 value for isoprenaline was 8.8 ± 0.8 ($n = 4$) and for D600 it was 6.3 ± 0.3 ($n = 3$). None of the prostanoids BW 245c (up to 30 μmol l⁻¹), ZK110841 (up to 10 μmol l⁻¹), AH 13205 (up to 80 μmol l⁻¹) or cicaprost (up to 0.14 μmol l⁻¹) produced detectable inhibition of activity.

Discussion

The similar and relatively high potencies with which the natural prostanoids PGD₂, PGE₂ and PGF_{2α} caused contractions of the myometrium of nonpregnant ewes *in vitro* suggest that each agent produces its effect by action at its own receptor (Coleman *et al.*, 1990): the DP, EP, and FP receptors, respectively. However, some evidence suggests that DP receptors are not present in sheep myometrium. First, DP receptors mediate predominantly inhibitory responses, for example, inhibition of smooth muscle contractions and platelet aggregation (Coleman *et al.*, 1990), rather than excitation. Second, the contractile effects of PGD₂ are not antagonised by the potent and selective DP receptor antagonist, BW A868c (Giles *et al.*, 1989). Third, the selective DP receptor agonists, BW245c (Town *et al.*, 1983) and ZK110841 (Thierauch *et al.*, 1988) are ineffective. The contractile effects of PGD₂ may be produced by action at EP, FP or TP receptors; however, its potency would be expected to be significantly less than those of PGE₂, PGF_{2α} or U46619, respectively (Coleman *et al.*, 1990). This is

Table 2. Effect of excitatory agonists on the contractility of the myometrium from nonpregnant ewes *in vitro*

Class	Compound	pD_2	SD	<i>n</i>
Natural prostanoid	PGD ₂	6.87	0.31	7
	PGE ₂	6.67	0.26	14
	PGF _{2α}	6.90	0.46	10
EP agonist	17-phenyltrilor PGE ₂	7.39	0.35	13
	GR 63799x	6.95	0.53	13
	Iloprost	8.34	0.12	4
	Misoprostol	8.31	0.4	4
	Sulprostone	7.08	0.26	9
	Cloprostenol	6.78	0.68	10
TP agonist	U46619	6.21	0.39	8
Non-prostanoid	Carbachol	6.68	0.36	8
	Oxytocin	9.22	0.42	8

Fig. 4. Effect of (a) time and (b) the cumulative addition of isoprenaline, on contractile activity induced by cloprostenol of the sheep myometrium *in vitro*. Contractile activity was determined during successive 10 min collection periods, the first of which represented the control. In (b) cumulative concentrations of isoprenaline in the bath for collection 1–11 were: 0, 0.01, 0.04, 0.14, 0.44, 1.44, 4.44, 14.4, 44.4, 144.4, 444 nmol l⁻¹, respectively. Zero contractile activity was that recorded in the presence of 100 μmol l⁻¹ D600. Data from (b) were corrected for the time-dependent changes in (a) to obtain a concentration–effect curve for isoprenaline.

not the case, and these results suggest that sheep myometrium contains an as yet uncharacterized excitatory receptor for PGD₂ (Ito *et al.*, 1990).

The presence of EP receptors in sheep myometrium is suggested by the values of the potencies of 17-phenyltrilor

PGE₂, GR63799x, iloprost, misoprostol and sulprostone in causing contraction of this tissue *in vitro*. However, there are four subtypes of EP receptors: EP₁ and EP₃ receptors mediate excitation of smooth muscle, whereas EP₂ and EP₄ receptors mediate inhibition (Coleman *et al.*, 1990, 1994).

The effectiveness and potency of sulprostone at provoking contractions of the sheep myometrium *in vitro* confirm the presence of excitatory EP receptors, since this compound has a high selectivity for EP₁ and EP₃ receptors (Coleman *et al.*, 1990). GR63799x is a selective agonist for EP₃ receptors; in EP₃ receptor-dominated preparations, it was 10–50 times more potent than PGE₂; and in an EP₁ dominated preparation, it was four times less potent (Bunce *et al.*, 1990). In this study, GR63799x was equipotent with PGE₂. This observation alone permits no definite conclusion about the site of action of GR63799x in the sheep myometrium. However, the observation that the selective EP₁ antagonist, AH6809, had no effect on the response to GR63799x strongly indicates that if EP₁ receptors are involved, they do not predominate. Thus, by default, the response to the agonist must be mediated mainly by EP₃ receptors. Consequently, we conclude that the sheep myometrium *in vitro* expresses EP₃ receptors linked to contraction. This conclusion is supported by the high contractile potency of misoprostol: a compound with mixed EP₂ and EP₃ activity (Coleman *et al.*, 1988).

The failure of the selective EP₂ agonist AH13205 (Nials *et al.*, 1991) to display either excitatory or inhibitory actions on the sheep myometrium suggests that EP₂ receptors are absent.

Iloprost has agonist activity at both EP₁ and IP receptors (Coleman *et al.*, 1990). Action at IP receptors would be expected to produce inhibition of smooth muscle contractility (Coleman *et al.*, 1990). Therefore, the potent contractile activity of iloprost suggests an action at EP₁ receptors. This is confirmed by the observation that iloprost was antagonised by AH6809. It has been suggested that 17-phenyltrinor PGE₂ is a selective EP₁ receptor agonist (Lawrence *et al.*, 1989), although the evidence to support this is not compelling, so its potency on the sheep myometrium might be taken as confirmation of the presence of EP₁ receptors in this preparation. However, unlike that of iloprost, the action of 17-phenyltrinor PGE₂ was not sensitive to AH6809, and therefore not mediated by EP₁ receptors. This observation highlights the danger of relying solely on selective agonist potencies for receptor classification.

There is, unfortunately, no choice but to rely upon the equipotency of PGF_{2α} and cloprostenol (Dukes *et al.*, 1974), a selective FP receptor agonist (Coleman *et al.*, 1990) to support the existence of FP receptors coupled to contraction in the sheep myometrium. Similar evidence using fluprostenol, an analogue of cloprostenol, has been used in the same way in human myometrium from both nonpregnant and term pregnant donors (Senior *et al.*, 1992, 1993).

Cicaprost shows a high degree of selectivity for the IP receptor (Coleman *et al.*, 1990). The fact that it was devoid of activity in our experiments suggests the absence of IP receptors in the sheep myometrium. This hypothesis is supported by the fact that we could detect no inhibitory action for iloprost, which is a potent IP receptor agonist (Coleman *et al.*, 1990). However, Lye and Challis (1982) demonstrated inhibition of myometrial activity *in vivo* by both PGI₂ and PGI₂ methyl ester in nonpregnant ovariectomized ewes; this observation suggests a role for the IP receptor in myometrial inhibition. Perhaps the explanation for the discrepancy is methodological. Either the different hormonal environment of ovariectomized versus seasonally oestrous ewes induces IP receptors in the myometrium, or PGI₂ acts indirectly to produce myometrial inhibition.

The activity of U46619, a stable synthetic thromboxane mimetic (Coleman *et al.*, 1990), suggests the presence of TP receptors in the sheep myometrium. This hypothesis is supported by the effectiveness of the selective TP antagonist L670596 (Ford-Hutchinson *et al.*, 1989) in blocking the responses to U46619. We determined a pK_B for L670596 of 8.4 in the sheep myometrium, whereas Ford-Hutchinson *et al.* (1989) report a value of 9.0 in the guinea-pig tracheal chain. This difference could be explained by action of U46619 at more than one type of receptor in the sheep myometrium, or by species variants of the TP receptor between sheep and guinea-pigs.

Our data are consistent with the hypothesis that the myometrium of the nonpregnant sheep contains all currently known excitatory prostanoid receptors: EP₁, EP₂, FP and TP, but no inhibitor receptors. Failure to demonstrate inhibition by the prostanoids was not methodological, since a clear concentration-dependent inhibition was produced by the β-adrenoceptor agonist isoprenaline.

The human myometrium also contains all excitatory receptors, but in addition, it contains DP, EP₂ and IP receptors (Senior *et al.*, 1992, 1993). Guinea-pig and rat uteri, like that of the sheep, lack inhibitory receptors but, unlike the sheep uterus, the guinea-pig uterus also lacks FP receptors and the rat uterus lacks TP receptors (Coleman *et al.*, 1990).

The pharmacological characterization of prostanoid receptors in the myometrium of the nonpregnant ewe performed in this study should help us to understand better the physiological role of prostanoids in reproductive processes in sheep.

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