

An investigation of the effect of the prostaglandin EP₂ receptor agonist, butaprost, on the human isolated myometrium from pregnant and non-pregnant women

N Duckworth, K Marshall and J K Clayton¹

School of Pharmacy, University of Bradford, Bradford, West Yorkshire, BD7 1DP, UK

¹Bradford Royal Infirmary, Duckworth Lane, Bradford, West Yorkshire, BD9 6RJ, UK

(Requests for offprints should be addressed to K Marshall; Email: K.M.Marshall@bradford.ac.uk)

Abstract

The aim of this study was to compare the effect of two known spasmogens, oxytocin and the stable thromboxane receptor mimetic, U46619, on human myometrium treated with the prostaglandin E receptor (EP₂) agonist, butaprost (selective for the EP₂ receptor).

Strips of myometrium from pregnant and non-pregnant donors were set up in a superfusion apparatus. Butaprost was administered as a bolus dose and via infusion. During the infusion of 10⁻⁶ M butaprost, spasmogens were administered as bolus doses.

Butaprost caused dose-related inhibition of myometrial activity when administered as a bolus dose (3–100 nmol) and concentration-dependent inhibition during infusion studies (10⁻⁸–10⁻⁵ M). Butaprost (10⁻⁶ M) attenuated

the response to U46619 (0.1–10 nmol) in pregnant myometrium, but this difference was not statistically significant. Responses of pregnant myometrium to oxytocin (0.001–0.1 nmol) were significantly attenuated ($P < 0.05$) in the presence of butaprost (10⁻⁶ M).

Butaprost physiologically antagonised the oxytocin response, possibly by increasing intracellular cAMP levels. This antagonism was much more marked than that seen with butaprost and U46619. It is unclear why these two types of antagonism differ and this effect is currently being investigated further using other prostanoid and non-prostanoid agents.

Journal of Endocrinology (2002) **172**, 263–269

Introduction

The prostanoid receptors DP, EP, FP, IP and TP are classified according to their sensitivity to the five primary prostanoids (PG, prostaglandins): PGD₂, PGE₂, PGF_{2α}, PGI₂ and thromboxane A₂ (TXA₂) respectively (Coleman 1998). Furthermore, by using various selective agonists and antagonists, the EP receptor can be divided further into the subtypes EP₁, EP₂, EP₃ and EP₄ (Coleman *et al.* 1990). Of these four receptor subtypes, EP₃ and EP₄ are the most abundant throughout the human body, and Northern blotting has detected EP₃ and EP₄ mRNA in nearly all mouse tissues (Sugimoto *et al.* 1992). EP₁ and EP₃ receptors are considered excitatory, causing increases in phosphatidylinositol turnover and inhibition of adenylyl cyclase respectively. EP₂ and EP₄ receptors, on the other hand, increase intracellular cAMP levels by stimulating adenylyl cyclase activity (Coleman 1998).

EP₃ receptors are involved in uterine contraction whilst EP₂ receptors mediate inhibition of myogenic activity (see Fig. 1a) (Senior *et al.* 1991, 1993). This group also found a paucity of EP₁ receptors, especially in tissue from pregnant donors, which also mediate contraction.

Upon examination of the human uterus, it has been found to contain varying levels of EP₂, EP₃ (Senior *et al.* 1991, 1993) and EP₄ (Erkinheimo *et al.* 2000) receptors, dependent on the physiological state of the uterus, i.e. non-pregnant or pregnant. It has been proposed that the levels, and therefore the balance, of these receptors are a possible trigger in the initiation of labour (Matsumoto *et al.* 1997, Dong & Yallampalli 2000). Human studies showed a reduction of approximately 50% of EP₃ receptors during pregnancy as compared with non-pregnant myometrium (Matsumoto *et al.* 1997). EP₃ mRNA levels in sheep myometrium increase during labour whilst EP₂ mRNA levels remain the same (Ma *et al.* 1999). In human myometrium at term, there were no significant differences in EP₂ mRNA levels in tissue from women in labour compared with women not in labour (Brodt-Eppley & Myatt 1999).

As the levels of the EP₂ receptor purportedly remain constant, this receptor is a potential therapeutic target in the treatment of certain pathological states, for example, pre-term labour and dysmenorrhoea. Butaprost is a selective EP₂ agonist (Gardiner 1986) which mediates inhibition of myogenic activity through stimulation of adenylyl cyclase (Asboth *et al.* 1998).

The aim of this study was to investigate further the tocolytic effects of butaprost on myometrium taken from pregnant and non-pregnant donors and to challenge this induced quiescence with known spasmogens, namely oxytocin and the stable thromboxane mimetic U46619. Oxytocin and the oxytocin receptor have been widely researched in the area of pre-term labour (Fuchs *et al.* 1984, 1991) and it has been postulated that oxytocin itself may be a trigger in pre-term labour. U46619 is a selective TP agonist (Coleman *et al.* 1981) and it is known that there is a population of TP receptors on myometrium taken from both pregnant and non-pregnant donors, mediating smooth muscle contraction (Senior *et al.* 1992, 1993).

Methods and Materials

Methods

Myometrium The method used in the study has received Ethics Committee approval and all patients gave informed, written consent to donate their tissue before any surgical procedure was performed. All samples were collected from Bradford Royal Infirmary.

Samples of myometrium from non-pregnant (NP) donors were obtained at hysterectomy for benign disorders, predominantly menorrhagia. All non-pregnant samples were pooled, regardless of the stage of the menstrual cycle of the donors, as in this study (see also Senior *et al.* 1991) there was no significant difference in the functional responsiveness of the myometrium to the prostanoids used in these experiments. Segments of myometrium were taken from the anterior wall of the corpus uteri. Tissue from pregnant (P) women was taken from the lower segment of the uterus from women undergoing elective Caesarean section at 38–40 weeks gestation (but not in labour). Samples were placed in flasks containing Krebs solution, at room temperature, and were transported to the laboratory. The initial tissue preparatory procedures were completed within 1 h of surgery and were conducted as previously described by Senior *et al.* (1991). Briefly, longitudinal myometrial strips (20 × 3 × 3 mm), free from endometrium and serosa, were cut and placed under 2 g tension for superfusion with oxygenated Krebs (95% O₂/5% CO₂) containing 1 μM indomethacin (Coleman *et al.* 1990) at 37 °C. The myometrium was left to equilibrate for 2 h or until regular spontaneous activity had been established. The spasmogens (oxytocin and U46619) and butaprost were administered as bolus doses, injected directly into the superfusate, immediately after a spontaneous contraction. The profile of spontaneous activity and sensitivity to agonists often changed markedly throughout the course of the experiments and, therefore, agonist dose–effect curves were only carried out once on each strip of myometrium.

On examination of the traces, it was seen that the greater the magnitude of background contractions, the

greater the magnitude of excitatory response. Thus, the results were expressed as a T/B ratio, that is, test (T) agonist response (units of area tension) as a ratio of background (B) activity. The use of T/B ratio as an expression of agonist responses allows for the normalisation of the data, and a T/B ratio greater than zero demonstrates an excitatory drug effect (Senior *et al.* 1991). Agonist potency was assigned an ED₁ value – the dose required to cause a response of equal magnitude to background activity, i.e. at the level where T/B equalled 1. ED₁ values were obtained from individual dose–effect curves and expressed as arithmetic means with ranges (Senior *et al.* 1991). Each *n* value represents data from one strip of myometrium taken from a different donor for *n* experiments, for example, if *n*=7, 7 strips of myometrium would have been used, each strip coming from a different individual donor.

Butaprost was also infused into the tissue at a rate of 0.02 ml/min at varying concentrations between 0.01 and 10 μM. Recovery time was defined as the number of minutes taken for normal spontaneous activity to be resumed after a 15-min infusion period.

Measurement of responses Responses were measured via isometric transducers (Dyanometer UF1) linked to a balanced Grass Polygraph (Model 7D) chart pen recorder which showed tension changes in the tissue. The area of tension change was measured using BBC Digit software.

Statistical analysis Drug and dose data were analysed by a 2-way ANOVA with drug as an orthogonal variable and dose as a repeated measure (i.e. within subject) variable. In order to circumvent possible problems associated with violations of sphericity that may occur with repeated measures designs, and to prevent the inflated degrees of freedom generated by such designs, a Greenhouse–Geisser correction was applied. The degrees of freedom for the error terms are set at *K*-1 (groups) and *N*-1 (total number of tissue samples tested). Differences were considered significant when *P*<0.05 (Bray & Maxwell 1982).

Materials

Butaprost (15-deoxy-16-hydroxy,17-cyclobutyl PGE₁) was obtained from Bayer Ltd (Newbury, Berks, UK). U46619 (11 α ,9 α -epoxymethano PGH₂) and indomethacin were obtained from Sigma-Aldrich Chemical Co. (Poole, Dorset, UK). PGE₂ was acquired from Cayman Chemical (distributed by Alexis Corporation (UK) Ltd, Bigham, Notts, UK) and oxytocin (Syntocinon) from Sandoz Pharmaceuticals (Camberley, Surrey, UK). Dilutions were made with 0.9% (w/v) normal saline.

Results

Unlike the parent prostaglandin, PGE₂ (see Fig. 1a), butaprost only evoked a monophasic, inhibitory response

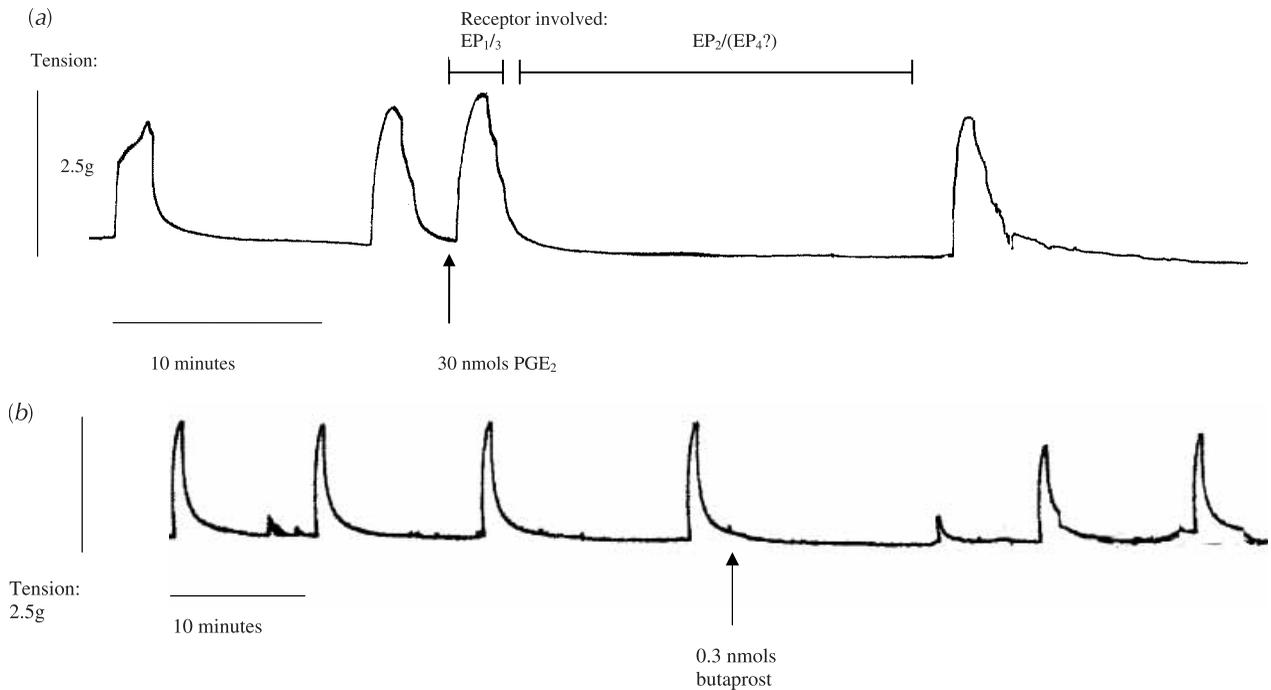


Figure 1 (a) Sample trace showing the biphasic response to a bolus dose of prostaglandin (PG)_E₂ on myometrium from pregnant donors. (b) Sample trace showing the monophasic response to a bolus dose of butaprost on myometrium from pregnant donors.

in P and NP human myometrium (Fig. 1b). Human myometrial activity (P and NP) was inhibited in a dose-dependent manner (3 nmol–100 nmol) by butaprost (Fig. 2). Butaprost had a significantly greater effect at mediating inhibition on myometrium from pregnant donors compared with that from non-pregnant women ($F[1,13]=5.2$, $P<0.05$)

After a 15-min infusion of butaprost, the time taken for normal myogenicity to be re-established also increased in

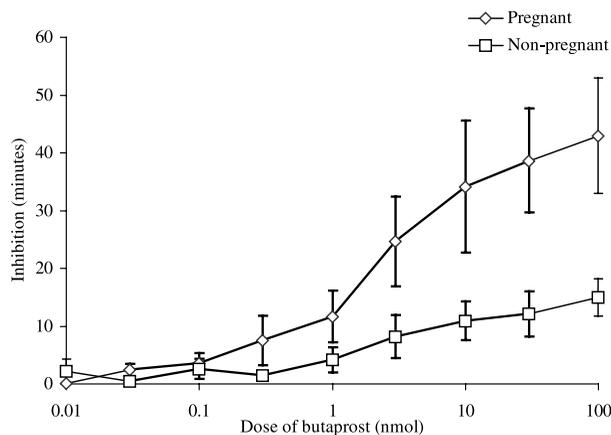


Figure 2 Mean inhibitory dose-effect curves to butaprost on pregnant and non-pregnant myometrium ($n=7$ in both instances). Data are expressed as arithmetic means and vertical bars represent S.E.M.

a concentration-dependent manner (Fig. 3). Using an infusion concentration of 10^{-6} M butaprost, the recovery time was 75 min and 82 min in myometrium from pregnant and non-pregnant donors respectively. At concentrations above 10^{-6} M, the recovery time exceeded 2 h in both tissues. The effect of infusion of 10^{-6} M butaprost

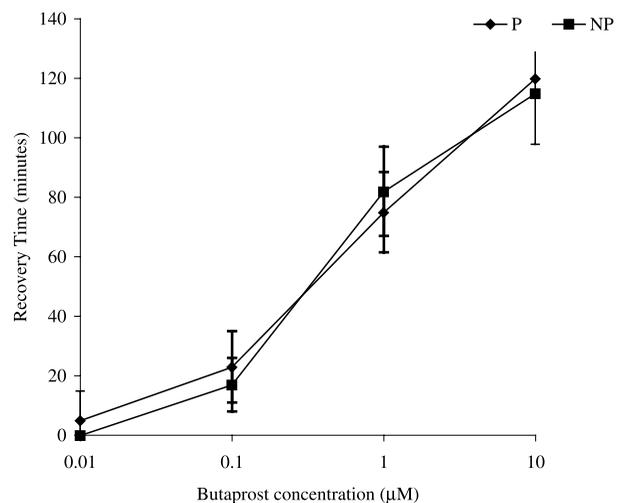


Figure 3 Time taken for normal myogenic activity of pregnant (P) and non-pregnant (NP) myometrium to be re-established after a 15-min infusion of butaprost (0.01–10 µM). Data are expressed as arithmetic means ($n=6$) and vertical bars represent S.E.M.

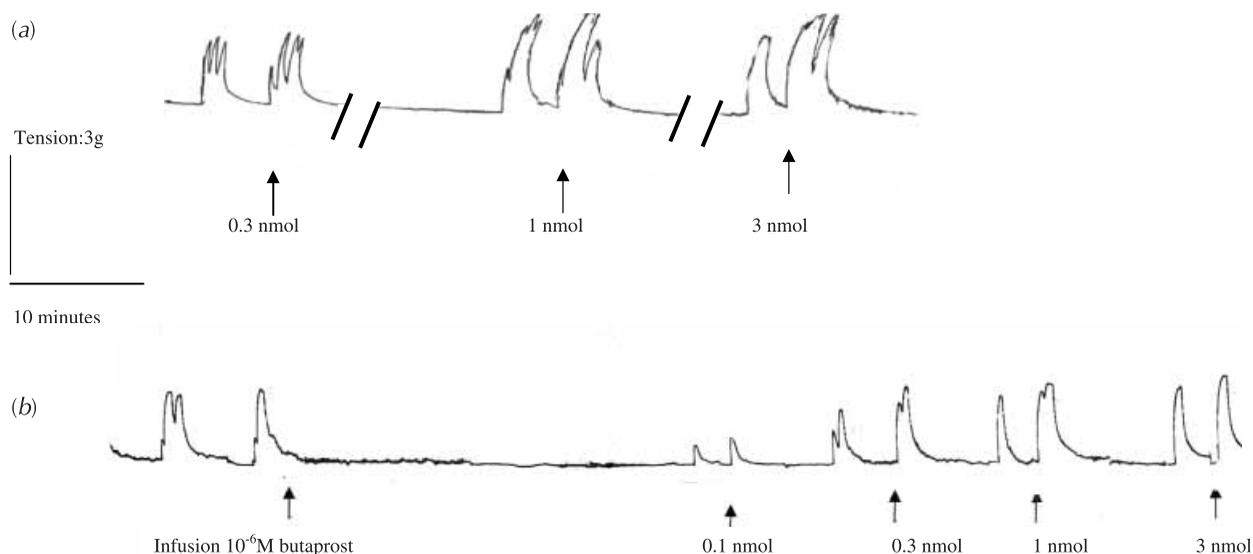


Figure 4 Sample traces showing activity of pregnant myometrium to bolus doses of U46619 alone (a) and in the presence of 10^{-6} M butaprost (b).

allowed sufficient time for restoration of myogenicity and therefore it was selected as the concentration which would be challenged with known uterotonins.

This inhibition of myometrial (P) activity was subsequently challenged with two known spasmogens, U46619 and oxytocin (Figs 4–7). Both U46619 and oxytocin were potent contractile agents and the U46619-induced responses were dose related. This relationship was seen with oxytocin up to a dose of 0.03 nmol, after which the response was attenuated. Oxytocin was the most potent

uterotonin tested, eliciting an effect at 0.001 nmol (Figs 5 and 7), whereas an effect was seen at 0.1 nmol with U46619 (Figs 4 and 6). Inhibition of NP activity by butaprost (10^{-6} M) was also challenged by U46619 (data not shown). U46619 induced a dose-related excitatory response (0.01–10 nmol) both alone and in the presence of butaprost (10^{-6} M). There was no statistically significant difference in either P or NP tissues between the responses to U46619 in the presence of butaprost as compared with U46619 alone. In contrast, in the presence of butaprost

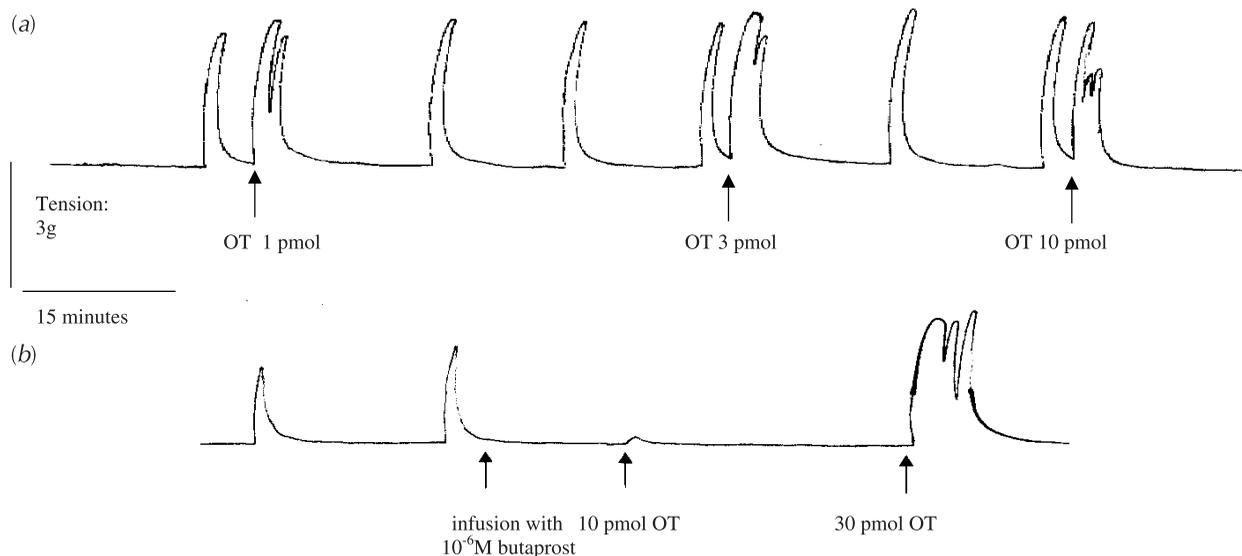


Figure 5 Sample traces showing activity of pregnant myometrium to bolus doses of oxytocin (OT) alone (a) and in the presence of butaprost 10^{-6} M (b).

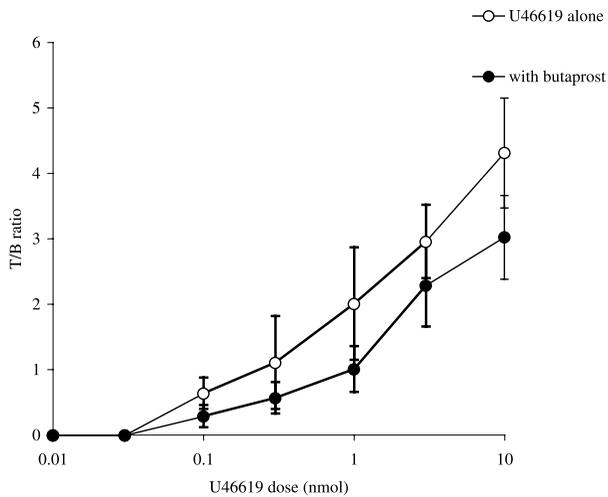


Figure 6 The effect of bolus doses of U46619 alone and in the presence of an infusion of butaprost (10^{-6} M) on isolated human myometrium ($n=6$) from pregnant donors. Vertical bars represent S.E.M.

(10^{-6} M), the response to oxytocin was significantly attenuated ($F[1,9]=5.27$, $P<0.05$) (Fig. 7).

Table 1 shows the ED₁ values obtained for oxytocin and U46619 in the absence and presence of butaprost (10^{-6} M). ED₁ values for U46619 alone (0.3 nmol) and in the presence of butaprost (1.3 nmol) were similar. However, the ED₁ value for oxytocin with butaprost (0.02 nmol) was approximately ten times greater than the value for oxytocin alone (0.0013 nmol), indicating the inhibitory potency of butaprost.

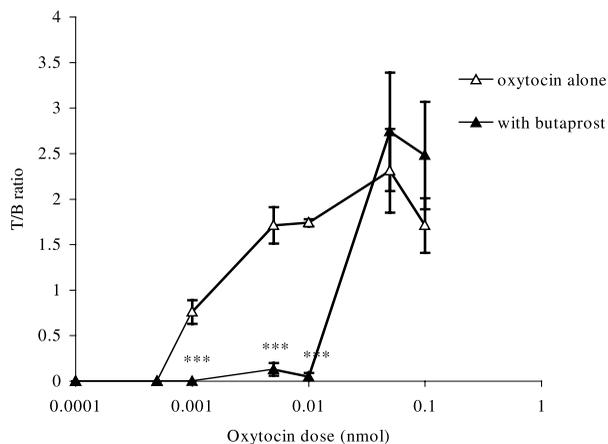


Figure 7 The effect of bolus doses of oxytocin alone and in the presence of an infusion of butaprost (10^{-6} M) on isolated human myometrium ($n=5$) from pregnant donors. Vertical bars represent S.E.M. and *** denote significant difference ($P<0.001$) compared with oxytocin alone.

Table 1 ED₁ values for oxytocin and U46619 alone and in the presence of butaprost (10^{-6} M), on tissues taken from pregnant and non-pregnant donors

Treatment	ED ₁ value (range)	
	Pregnant	Non-pregnant
U46619	0.3 (0.08–0.85)	0.39 (0.07–1)
Oxytocin	0.0013 (0.0007–0.0028)	ND
U46619+butaprost	1.3 (0.2–2.4)	0.2 (0.1–0.6)
Oxytocin+butaprost	0.02 (0.005–0.09)	ND

ND=not done – the response to oxytocin was not tested on non-pregnant myometrium as there are very few oxytocin receptors on this tissue as compared with pregnant myometrium (Kimura *et al.* 1996).

Discussion

The results confirm the presence of inhibitory EP₂ receptors in both P and NP myometrium. Stimulation of this receptor leads to an increase in adenylyl cyclase activity, therefore increasing intracellular cAMP levels (Asboth *et al.* 1998). The action of butaprost was significantly greater in P myometrium than in NP tissues which suggests there are more EP₂ receptors in P myometrium. This would be consistent with the physiological state of the uterus which, during gestation, remains quiescent. It should be noted that this tissue, although taken at term, was from elective Caesarean sections and that the women had not gone into labour. An important factor which must not be overlooked when comparing EP₂ receptor populations between P and NP myometrium is the site of excision of the tissue. Tissue from pregnant donors in this study was taken from the lower segment of the uterus (for ethical reasons) whilst NP myometrium was obtained from the anterior wall of the corpus uteri. It is possible that the receptor population varies down the length of the uterus. A study by Hofmann and colleagues (1983) showed that smooth muscle content is greater at the fundus, decreasing down the length of the uterus. This was accompanied by a decrease in binding of PGE₁ and PGF_{2 α} and this finding would support the theory that the topology of prostanoid receptor populations varies in the uterus. In relation to our results, the greater EP₂ population in P myometrium may indeed be due to its role in maintaining quiescence during pregnancy but it is conceivable that differences may be due to the lower smooth muscle content in these segments of uterus.

Towards term, prostaglandin FP receptors (which mediate contraction) are up-regulated (Matsumoto *et al.* 1997) and their activity dominates uterine activity. Large standard errors are observed in the dose–response curve to butaprost which may reflect the change in balance of excitatory to inhibitory receptors which takes place towards term. The mean gestation period was 38 weeks for P tissue; however, in some instances the up-regulation

of excitatory receptors may have begun. It is possible, therefore, that the hormone milieu from which the tissue was taken may influence receptor populations and, thus, tissue responsiveness to the given agonists.

When observing the effect of a 15-min infusion of butaprost, there is no significant difference in the inhibitory effects between P and NP tissues (Fig. 3). This lack of difference, as compared with bolus dosing, may be a reflection of the fact that equilibration is more likely to be achieved during infusion. Even if there are fewer receptors on NP tissue, this fact may be overcome when using the infusion technique, as butaprost is known to be highly lipid soluble and thus, under infusion conditions, it is more likely to form an active receptor–ligand complex and therefore mediate a response.

Stimulation of uterine contraction with U46619 and oxytocin corroborate the presence of TP and oxytocin receptors, respectively, on P tissue and TP receptors on NP tissues. The response to oxytocin was not tested on NP myometrium as there are very few oxytocin receptors on this tissue as compared with P myometrium (Kimura *et al.* 1996). In the presence of butaprost, the dose–response curve to U46619 was attenuated although not significantly in P myometrium (ED₁ values = 0.3 (U46619 alone) and 1.3 (with butaprost)). In NP tissues, the response to U46619 in the presence of 10⁻⁶ M butaprost was not significantly affected compared with the response to U46619 alone (ED₁ values: 0.39 (0.07–1) (alone) and 0.2 (0.1–0.6) (with butaprost)). Any antagonism at the prostaglandin receptor level is unlikely between these two agonists as butaprost and U46619 are generally accepted as selective EP₂ and TP receptor agonists respectively. However, in terms of any physiological antagonism this situation may be complicated further by the different sites of excision of the two tissues and the hormonal status of the donor: for example, the presence of more excitatory receptors in the upper segment of the uterus (Wikland *et al.* 1984) and the fact that other excitatory receptors such as the FP receptor (Brodt-Eppley & Myatt 1999) are known to be up-regulated as term approaches.

Oxytocin receptors are up-regulated during gestation (Kimura *et al.* 1996) and oxytocin sensitivity, at a given gestational age, is significantly increased in women who deliver prematurely compared with those women who deliver at term (Takahashi *et al.* 1980). In the present study, the response to oxytocin was significantly attenuated up to a dose of 0.03 nmol ($P < 0.05$) in P myometrium in the presence of butaprost (10⁻⁶ M). It is possible that this effect is caused by cAMP overriding the oxytocin-induced contractile response (phosphatidylinositol hydrolysis accompanied by the opening of calcium channels). When oxytocin is administered (0.03 nmol and above) there is a large increase of intracellular calcium (Thornton *et al.* 1992), calcium–calmodulin complexes are formed and myosin light chain kinase (MLCK) is phosphorylated, leading to contraction of uterine smooth

muscle. As butaprost can reduce oxytocin-induced contractions, it may have a therapeutic role as a tocolytic. In addition to this, there were no significant differences in EP₂ mRNA levels in myometrium taken from women not in labour compared with women in labour (Brodt-Eppley & Myatt 1999), therefore down-regulation would not reduce the tocolytic ability of butaprost. It must be noted, however, that the response to the stable thromboxane mimetic, U46619, was not significantly attenuated in the presence of butaprost. This may be attributable to the fact that both agonists are prostanoids. In the case of oxytocin and butaprost, it is unlikely that the antagonism occurs at the receptor due to the chemical nature of the molecules; it is more likely that physiological antagonism is occurring. What is unclear is why the physiological antagonism is significantly greater than the potential prostanoid receptor antagonism. However, it is unlikely that butaprost has any effect at the TP receptor as it is a known selective EP₂ receptor agonist (Coleman 1998). This aspect is currently being investigated further by observing the effect of butaprost on other prostanoid and non-prostanoid agents. Current studies are also focusing on second messenger systems, activated in prostanoid and non-prostanoid responses, which may explain the difference in the level of antagonism seen with these agents.

To conclude, the EP₂ receptor is a target for novel, potential tocolytic therapies due to its ability to reduce myogenic activity in pregnant myometrium and significantly to attenuate the response to the known spasmogen, oxytocin. What must be investigated further, if butaprost is to be considered as a potential tocolytic agent, is the ability of this EP₂ receptor agonist to attenuate prostanoid-like agonists, as these agents are also involved in parturition and labour.

References

- Asboth G, Phaneuf S & Lopez Bernal AL 1998 Prostaglandin E receptors in myometrial cells. *Acta Physiologica Hungarica* **85** 39–50.
- Bray JH & Maxwell SE 1982 Analyzing and interpreting significant MANOVAs. *Review of Educational Research* **52** 340–367.
- Brodt-Eppley J & Myatt L 1999 Prostaglandin receptors in lower segment myometrium during gestation and labor. *Obstetrics and Gynecology* **93** 89–93.
- Coleman RA 1998 Prostanoid Receptors. In *The IUPHAR Receptor Compendium*, pp 230–244. Compiled by the IUPHAR Committee on Receptor Nomenclature and Drug Classification. London: IUPHAR Media.
- Coleman RA, Humphrey PPA, Kennedy I, Levy GP & Lumley P 1981 Comparisons of the actions of U46619, a stable prostaglandin H₂ analogue, with those of prostaglandin H₂ and thromboxane A₂ on some smooth muscle preparations. *British Journal of Pharmacology* **73** 773–778.
- Coleman RA, Kennedy I, Humphrey PPA, Bunce KI & Lumley P 1990 Prostanoids and their receptors. In *Comprehensive Medicinal Chemistry*, vol 3, pp 643–714. Eds C Hancsch & JC Emmett. Oxford: Pergamon Press.
- Dong YL & Yallampalli C 2000 Pregnancy and exogenous steroid treatments modulate the expression of relaxant EP(2) and contractile FP receptors in the rat uterus. *Biology of Reproduction* **62** 533–539.

- Erkinheimo TL, Saukkonen K, Narko K, Jalkanen J, Ylikorkala O & Ristimäki A 2000 Expression of cyclooxygenase-2 and prostanoid receptors by human myometrium. *Journal of Clinical Endocrinology and Metabolism* **85** 3468–3475.
- Fuchs A-R, Fuchs F, Husslein P & Soloff MS 1984 Oxytocin receptors in the human uterus during pregnancy and parturition. *American Journal of Obstetrics and Gynecology* **150** 734–741.
- Fuchs A-R, Romero R, Keefe D, Parra M, Oyarzum E & Behnke E 1991 Oxytocin secretion and human parturition: pulse frequency and duration increase during spontaneous labor in women. *American Journal of Obstetrics and Gynecology* **165** 1515–1523.
- Gardiner M 1986 Characterisation of prostanoid relaxant/inhibitory receptors using a highly selective agonist, TR4979. *British Journal of Pharmacology* **87** 45–56.
- Hofmann GE, Rao CV, Barrows GH & Sanfilippo JS 1983 Topography of human uterine prostaglandin E and F_{2α} receptors and their profiles during pathological states. *Journal of Clinical Endocrinology and Metabolism* **57** 360–366.
- Kimura T, Takemura M, Nomura S, Nobunaga T, Kubota Y, Inoue T, Hashimoto K, Kumazawa I, Ito Y, Ohashi K, Koyama M, Azuma C, Kitamura Y & Saji F 1996 Expression of oxytocin receptor in human pregnant myometrium. *Endocrinology* **137** 780–785.
- Ma X, Wu WX & Nathanielsz PW 1999 Differential regulation of prostaglandin EP and FP receptors in pregnant sheep myometrium and endometrium during spontaneous term labor. *Biology of Reproduction* **61** 1281–1286.
- Matsumoto T, Sagawa N, Yoshida M, Mori T, Tanaka I, Mukoyama M, Kotani M & Nakao K 1997 The prostaglandin E₂ and F_{2α} receptor genes are expressed in human myometrium and are down-regulated during pregnancy. *Biochemical and Biophysical Research Communications* **238** 838–841.
- Senior J, Marshall K, Sangha R, Baxter GS & Clayton JK 1991 *In vitro* characterisation of prostanoid EP-receptors in the non-pregnant human myometrium. *British Journal of Pharmacology* **102** 747–753.
- Senior J, Sangha R, Baxter GS, Marshall K & Clayton JK 1992 *In vitro* characterisation of prostanoid FP-, DP-, IP- and TP-receptors in the non-pregnant human myometrium. *British Journal of Pharmacology* **107** 215–221.
- Senior J, Marshall K, Sangha R & Clayton JK 1993 *In vitro* characterisation of prostanoid receptors on human myometrium at term pregnancy. *British Journal of Pharmacology* **108** 501–506.
- Sugimoto Y, Namba T, Honda A, Hayashi Y, Negishi M, Ichikawa A & Narumiya S 1992 Cloning and expression of a cDNA for mouse prostaglandin E receptor EP₃ subtype. *Journal of Biological Chemistry* **267** 6463–6466.
- Takahashi K, Diamond F, Bieniarz J, Yen H & Burd L 1980 Uterine contractility and oxytocin sensitivity in preterm, term and postterm pregnancy. *American Journal of Obstetrics and Gynecology* **136** 774–779.
- Thornton S, Gillespie JI, Greenwell JR & Dunlop W 1992 Mobilization of calcium by the brief application of oxytocin and prostaglandin E₂ in single cultured human myometrium cells. *Experimental Physiology* **77** 293–305.
- Wikland M, Lindholm B & Wiqvist N 1984 Myometrial response to prostaglandins during labor. *Gynecological and Obstetric Investigation* **17** 131–138.

Received in final form 14 September 2001
Accepted 8 October 2001