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Control of cyclic AMP concentration in bovine endometrial stromal cells by arachidonic acid

Z Cheng, E L Sheldrick, E Marshall, D C Wathes[1], D R E Abayasekara[1] and A P F Flint

School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK and [1]Reproduction, Genes and Development Group, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts AL9 7TA, UK

Correspondence should be addressed to A P F Flint; Email: anthony.flint@nottingham.ac.uk

Z Cheng is now at Laboratory of Stem Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-yang Road, Shanghai 200031, People's Republic of China

Abstract

Second messenger signalling through cyclic AMP (cAMP) plays an important role in the response of the endometrium to prostaglandin (PG) E_2 during early pregnancy. Arachidonic acid, which is a by-product of the luteolytic cascade in ruminants, is a potential paracrine signal from the epithelium to the stroma. We investigated the effects of arachidonic acid on the response of the stroma to PGE_2. cAMP was measured in bovine endometrial stromal cells treated with agents known to activate or inhibit adenylyl cyclase, protein kinase C (PKC) or phosphodiesterase (PDE). PGE_2 increased the intracellular cAMP concentration within 10 min, and this effect was attenuated by arachidonic acid and the PKC activator, 4β-phorbol myristate acetate (PMA). The inhibitory effect of arachidonic acid on PGE_2-induced cAMP accumulation was prevented by the PKC inhibitor, RO318425, and was absent in cells in which PKC had been downregulated by exposure to PMA for 24 h. The effect of arachidonic acid was also prevented by the PDE inhibitor, 3-isobutyl-1-methylxanthine. Arachidonic acid was shown by immunoblotting to prevent induction of cyclooxygenase-2 by PGE_2, forskolin or dibutyryl cAMP. The results indicate that arachidonic acid activates PDE through a mechanism involving PKC, counteracting a rise in intracellular cAMP in response to PGE_2. The data suggest that arachidonic acid antagonizes PGE_2 signalling through cAMP in the bovine endometrium, possibly acting to ensure a rapid return to oestrus in the case of failure of the maternal recognition of pregnancy.

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Introduction

Intracellular signalling through adenylyl cyclase and 3′,5′-cyclic AMP (cAMP) plays an important role in the preparation of the uterus for implantation. Compounds activating adenylyl cyclase in the endometrium include prostaglandin (PG) E_2, relaxin, activin, luteinizing hormone and corticotrophin-releasing hormone, all of which have been implicated in changes in endometrial function during early pregnancy (Tseng *et al.* 1992, Tang & Gurpide 1993, Frank *et al.* 1994, Ferrari *et al.* 1995). Administration of dibutyryl cAMP to the uterine lumen in mice has been known for three decades to induce a decidual response and implantation (Webb 1977). Recently, it has been proposed that cAMP acts to support the action of progesterone, through synergy between the protein kinase A substrate, CREB and the progesterone receptor (Gellerson & Brosens 2003).

The role of PGE_2 in activating adenylyl cyclase at implantation has been particularly well defined in species with endometrial decidualization. In mouse, rat, rabbit and man, PGE_2 is involved in endometrial angiogenesis (Jabbour & Sales 2004), vasodilatation and vascular permeability (Kennedy 1983), stromal cell prolactin expression (Frank *et al.* 1994) and decidualization. These processes involve signalling through the PGE_2 receptors EP2 and EP4, which are linked to adenylyl cyclase (Fujino *et al.* 2005) and are expressed by endometrial epithelial and stromal cells.

Although they are less well understood, PGE_2 has similar effects in species without decidualization and in which implantation is superficial, such as ruminants. In the bovine uterus, epithelial and stromal cells express EP2 (but not EP4) receptors (Arosh *et al.* 2003); both cell types synthesize PGE_2 (Fortier *et al.* 1988, Asselin *et al.* 1996), and PGE synthase concentrations increase with

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time after ovulation (Arosh *et al.* 2002). The blastocyst also secretes PGE_2 in cattle (Wilson *et al.* 1992) and sheep (Marcus 1981, Hyland *et al.* 1982), and PGE_2 is luteotrophic on administration to the uterine lumen (Magness *et al.* 1981). As a result, PGE_2 has been suggested to function as a maternal recognition of pregnancy signal in these species. Thus, although all aspects of the involvement of PGE_2 and cAMP in early pregnancy have not been demonstrated in all species, they are clearly involved whether or not the endometrium decidualizes.

Genes transcribed in response to cAMP in the endometrial stroma have principally been studied in the context of decidualization (Popovici *et al.* 2000). Among these are genes coding for a variety of transcription factors, growth factors and compounds involved in angiogenesis, and the decidualization marker prolactin (Christian *et al.* 2002, Gellerson & Brosens 2003, Yoshino *et al.* 2003). They also include cyclooxygenase-2 (COX-2; Zhou *et al.* 1999, Schroer *et al.* 2002, Wu & Wiltbank 2002), which plays an essential role in the establishment of pregnancy (Reese *et al.* 2001) by converting arachidonic acid into prostanoids required for implantation (Lim *et al.* 1999). As COX-2 is induced by cAMP and produces the substrate for PGE synthase, and as PGE_2 activates adenylyl cyclase, a positive feedback loop has been proposed whereby PGE_2 induces its own synthesis (Sales *et al.* 2001, Arosh *et al.* 2004).

Intracellular concentrations of cAMP are determined not only by its synthesis, but also by its catabolism to 5′-AMP, which is catalysed by a member of the phosphodiesterase (PDE) family specific for cAMP (cAMP-PDE). cAMP-PDE is activated by phosphorylation, notably by protein kinase C (PKC; Tetsuka *et al.* 1995, Cai & Lee 1996, Geoffroy *et al.* 1999, Bian *et al.* 2000). Compounds activating PKC might, therefore, be expected to reduce intracellular levels of cAMP, and so may oppose the action of PGE_2 and hinder the establishment of pregnancy.

Arachidonic acid and other polyunsaturated fatty acids are PKC activators (Khan *et al.* 1995), which may lead to activation of cAMP-PDE. Arachidonic acid is produced in the endometrium at luteolysis through cytosolic phospholipase A_2 (Lee & Silvia 1994, Burns *et al.* 2000) and in response to activation of phospholipase C following oxytocin receptor (OTR) occupancy (Flint *et al.* 1986), and has been proposed to act as a paracrine messenger between the endometrial epithelium and stroma at luteolysis (Sheldrick *et al.* 2006), as in other reproductive tissues (Cooke *et al.* 1991). Both OTR concentrations and PKC activity were higher in the non-pregnant horns of unilaterally pregnant ewes on day 16 after oestrus than they were in the pregnant horns of the same sheep (Abayasekara *et al.* 1995), which is consistent with oxytocin-induced production of PKC activators such as diacylglycerol or arachidonic acid. Control of arachidonic acid release from cells through endocrine or immune stimulation is consistent with paracrine or autocrine functions of fatty acids in other tissues (Zheng *et al.* 1999, Ronco *et al.* 2002).

In addition to PGE_2, COX-2 also provides the substrate for $PGF_{2\alpha}$ production, the ratio PGE_2:$PGF_{2\alpha}$ produced being determined by the relative activities of PGE and PGF synthases. Through $PGF_{2\alpha}$ (FP) receptor activation of phospholipase C, $PGF_{2\alpha}$ also induces PKC (Abayasekara *et al.* 1993), and therefore, metabolites of arachidonic acid may also affect cAMP-PDE activity.

The experiments described here were designed to determine whether arachidonic acid interferes with cAMP signalling in bovine endometrial stromal cells, by measuring cAMP in cells in culture and using COX-2 levels as an indicator of the cellular response to cAMP signalling.

Materials and Methods

Materials

Unless otherwise stated, all compounds were obtained from Sigma or Calbiochem (Nottingham, UK). Arachidonic acid was stored under N_2 at −20 °C in darkness. Arachidonic acid, PGE_2 and 3-isobutyl-1-methylxanthine (IBMX) were added to culture media in ethanol; 4β-phorbol myristate acetate (PMA) and RO318425 were added in dimethylsulphoxide. Control cultures contained the vehicle as appropriate.

Cell culture

Bovine uterine stromal (BST) cells isolated from one uterine horn of a non-pregnant cyclic cow on day 16 post-oestrus (Flint *et al.* 2002) were maintained in Dulbecco's modified Eagles medium (DMEM; Sigma) with 1% antibiotic–antimycotic (ABAM; Sigma) and 10% foetal bovine serum at 37 °C, 95% humidity and 5% CO_2. They were passaged at intervals of 3–4 days when about 80% confluent. The stromal (as opposed to epithelial) phenotype of the cells was confirmed by their prostanoid secretion pattern ($PGE_2 > PGF_{2\alpha}$; Asselin *et al.* 1996). For experimental treatments, cells were plated into multiwell plates 48–72 h before use. The medium was changed to DMEM containing 10% dextran-coated charcoal-stripped foetal bovine serum and 1% ABAM at the time of addition of test compounds. For intracellular cAMP measurements, BST cells were seeded into six-well plates at a density of 10^6 cells/well; for immunoblotting cells were plated in 24-well plates at a density of $2–4 \times 10^5$ cells/well.

Immunoblotting

Lysates (10 μg protein) were subjected to electrophoresis using 10% acrylamide gels (5% stacking gels) before electroblotting onto Optitran BA-s 83 membrane (Schleicher and Schuell, Anderman and Company, Kingston-upon-Thames, UK) in 25 mmol/l Tris (pH 8.3) containing 148 mmol/l glycine and 20% (v/v) methanol. For detection

of COX-2, membranes were probed with COX-2 antibody (C-20; SC 1745, Santa Cruz, obtained through Autogen Bioclear, Calne, UK; 1:250 dilution in phosphate-buffered saline containing 1% w/v Marvel milk powder and 0.5% v/v Tween 20). The second antibody was donkey anti-goat IgG–horseradish peroxidase (SC 2020; Santa Cruz; 1:11 000 dilution in phosphate-buffered saline containing 3% w/v Marvel, 0.5% v/v Tween 20), and visualization was by ECL (Amersham) using Kodak BioMax Light film. Colour Markers (29–205 kD; Sigma) were used to identify molecular weights of proteins, and band intensities were quantified using Kodak 1D digital image analysis software.

3′,5′-cAMP assay

After incubation with different treatments, the medium was removed and the cells were treated with 0.1 mmol/l HCl to inhibit PDE activity and lysed with 0.1% v/v Triton X-100 for 10 min. The cAMP released was measured using either cAMP (low pH) immunoassay kits or the Parameter cAMP assay system (both from R&D Systems, Abingdon, UK) according to manufacturer's guidelines. Protein contents of lysates were measured to confirm that the wells contained equal numbers of cells; since this was consistently the case, cAMP concentrations were expressed as pmol cAMP/well.

Protein assay

Protein concentrations were measured in cell lysates by the BCA method (Perbio, Cramlingham, UK).

Experimental design and analysis

Experiments involved treatments carried out with at least three replicates (for cAMP measurement) or four replicates (for immunoblotting). All experiments were performed at least twice. To account for differences in band intensity between immunoblots, all blots included two control samples and all experimental treatments were related to the control bands. Statistical analysis of treatment effects was performed by analysis of variance using Genstat, with treatment and experiment identifier as factors. Where significant ($P<0.05$) effects were detected, individual means were tested by Student's *t*-test. Values are given as means ± S.E.M., and in the figures, bars with different letters are significantly different.

Results

PGE_2 increases cAMP concentration in BST cells

To confirm that PGE_2 activated adenylyl cyclase in BST cells, cAMP was measured in cells exposed to PGE_2 for varying times. As expected from the expression of EP2 receptors by bovine endometrium (Arosh *et al.* 2003), PGE_2 raised intracellular cAMP levels. In separate experiments, a 2.1-fold increase in cAMP was observed within 10 min of addition of PGE_2 and a threefold increase within 20 min (Fig. 1). None of the treatments affected cell protein concentration.

Inhibition by arachidonic acid of PGE_2-induced increase in cAMP concentration

To determine whether arachidonic acid affected PGE_2-induced cAMP accumulation, cells were treated with both PGE_2 and arachidonic acid. As shown in Fig. 2a, the increase in cAMP observed when cells were treated with PGE_2 was reduced by addition of arachidonic acid. Separate dose–response experiments showed that the concentration of arachidonic acid required to cause this effect was in the range of µmol/l (Fig. 2b).

Inhibition of PGE_2-induced cAMP accumulation by arachidonic acid is dependent on the PKC signalling pathway and requires activation of PDE

One possible mechanism by which arachidonic acid may block cAMP production is through PKC (Khan *et al.* 1995). This was confirmed using the PKC activator, 4β-PMA, which mimicked the effect of arachidonic acid (Fig. 3a). Furthermore, the inhibitory effect of arachidonic acid on the PGE_2-induced rise in cAMP level was blocked in cells cultured with the PKC inhibitor,

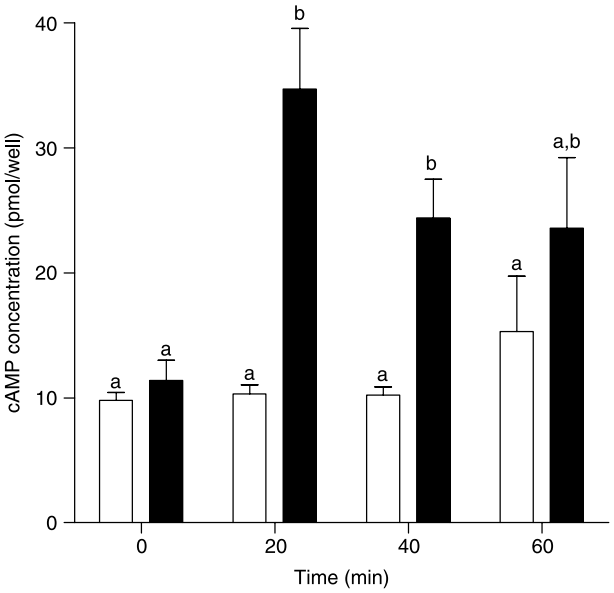

Figure 1 PGE_2 increased cyclic AMP concentration in bovine endometrial stromal (BST) cells. Cells were treated with PGE_2 (3 µmol/l) for various lengths of time before extraction for cyclic AMP assay. Open bars, control; closed bars, PGE_2. The cyclic AMP concentration was raised at 20 and 40 min ($P<0.05$), but the difference at 60 min was not statistically significant. In separate experiments, cyclic AMP levels were raised within 10 min of addition of PGE_2. In this and other figures, different superscript letters indicate significant effects.

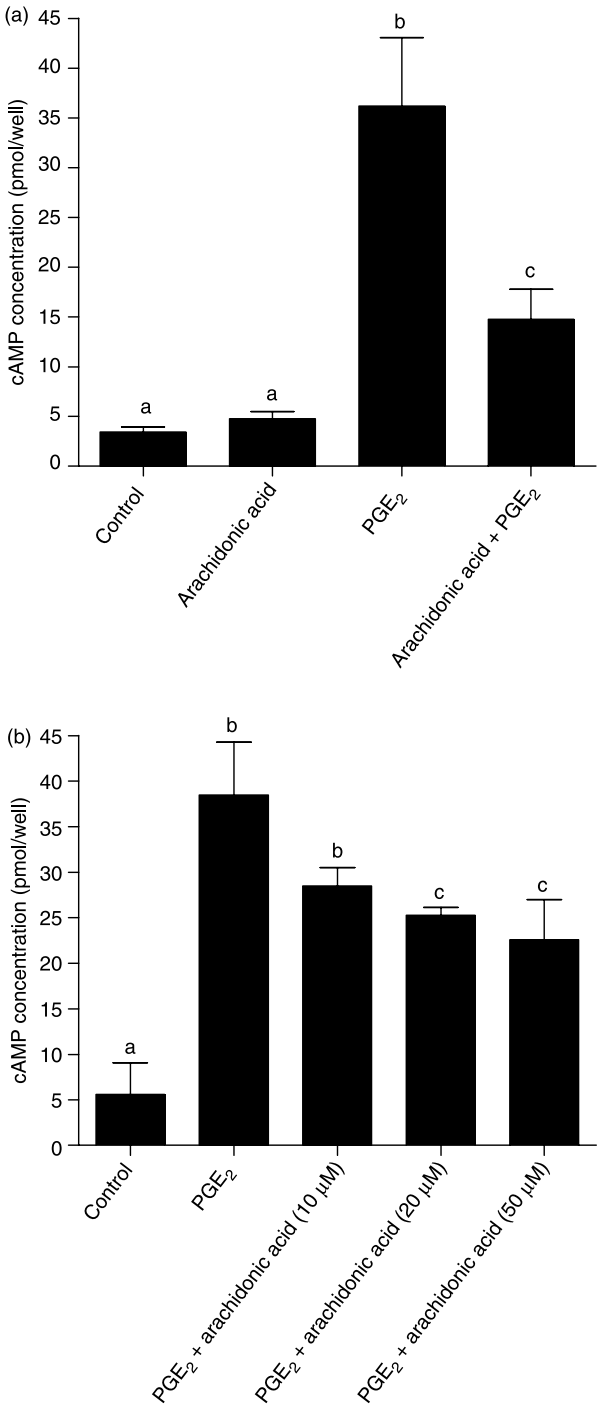

Figure 2 The effect of PGE_2 on intracellular cyclic AMP level was antagonized by arachidonic acid. (a) The intracellular concentration of cyclic AMP was increased by 3 μmol/l PGE_2 ($P<0.001$), but not by 50 μmol/l arachidonic acid alone. Arachidonic acid (50 μmol/l) reduced the level of cyclic AMP in cells treated with PGE_2 (3 μmol/l) for 20 min ($P<0.02$). (b) Dose–response experiments (carried out for 20 min) showed that arachidonic acid was effective ($P<0.05$) in the range of μmol/l.

RO318425 (Fig. 3b), and also by prolonged exposure of cells to 4β-PMA, which downregulates PKC (Akita *et al.* 1990, Kiley *et al.* 1990; Fig. 3c).

In view of the evidence from other cell types that PKC phosphorylates and thereby activates PDE isoforms responsible for metabolizing cAMP (Tetsuka *et al.* 1995, Cai & Lee 1996, Geoffroy *et al.* 1999, Bian *et al.* 2000), cells were cultured with the PDE inhibitor, IBMX. In the presence of 0.1 mmol/l IBMX, the inhibitory effect of arachidonic acid on PGE_2-induced cAMP accumulation was blocked (Fig. 3d). Identical effects were observed with 1 mmol/l IBMX (data not shown).

Effect of arachidonic acid on cAMP-induced COX-2 expression

To confirm that the effect of arachidonic acid on PDE was reflected in a cellular response to cAMP, COX-2 was measured by immunoblotting in cells treated with PGE_2 or dibutyryl cAMP with and without arachidonic acid. As anticipated (see Introduction), dibutyryl cAMP added alone increased COX-2 protein levels (Fig. 4a), and PGE_2 caused the same response (Fig. 4c). As expected on the basis of the activation of PDE, the increase in COX-2 level due to dibutyryl cAMP or PGE_2 was blocked by arachidonic acid. A similar observation was made with forskolin (1 mmol/l for 6 h), which increased COX-2 levels by 46% when added alone and by 7% when added with arachidonic acid (data not shown).

Discussion

Arachidonic acid antagonized the stimulatory effect of PGE_2 on cAMP levels in endometrial stromal cells. The effect of arachidonic acid required activation of PKC, since it was mimicked by the PKC activator, 4β-PMA, and blocked by the PKC inhibitor, RO318425, and downregulation of PKC. The effect appeared to be due to activation of cAMP-dependent PDE, since it was prevented by the PDE inhibitor, IBMX (although it should be noted that IBMX is not specific and other effects cannot be ruled out).

Activation of cAMP-dependent PDE by PKC is well known in other cell types, from studies of whole tissues (hamster heart, Lee *et al.* 1994; rat renal medullary collecting tubule, Tetsuka *et al.* 1995), intact cells (luteinizing human granulosa cells, Michael & Webley 1991; rat cardiac myocytes, Bian *et al.* 2000) and subcellular fractions (liver Golgi–endosomal fraction; Geoffroy *et al.* 1999). It occurs through a series of phosphorylation steps involving mitogen-activated protein kinase and PKA (Houslay & Adams 2003). There are many isoforms of PDE, as well as the kinases involved in this process, and it is not known which isoforms are present, or functional, in the bovine endometrium.

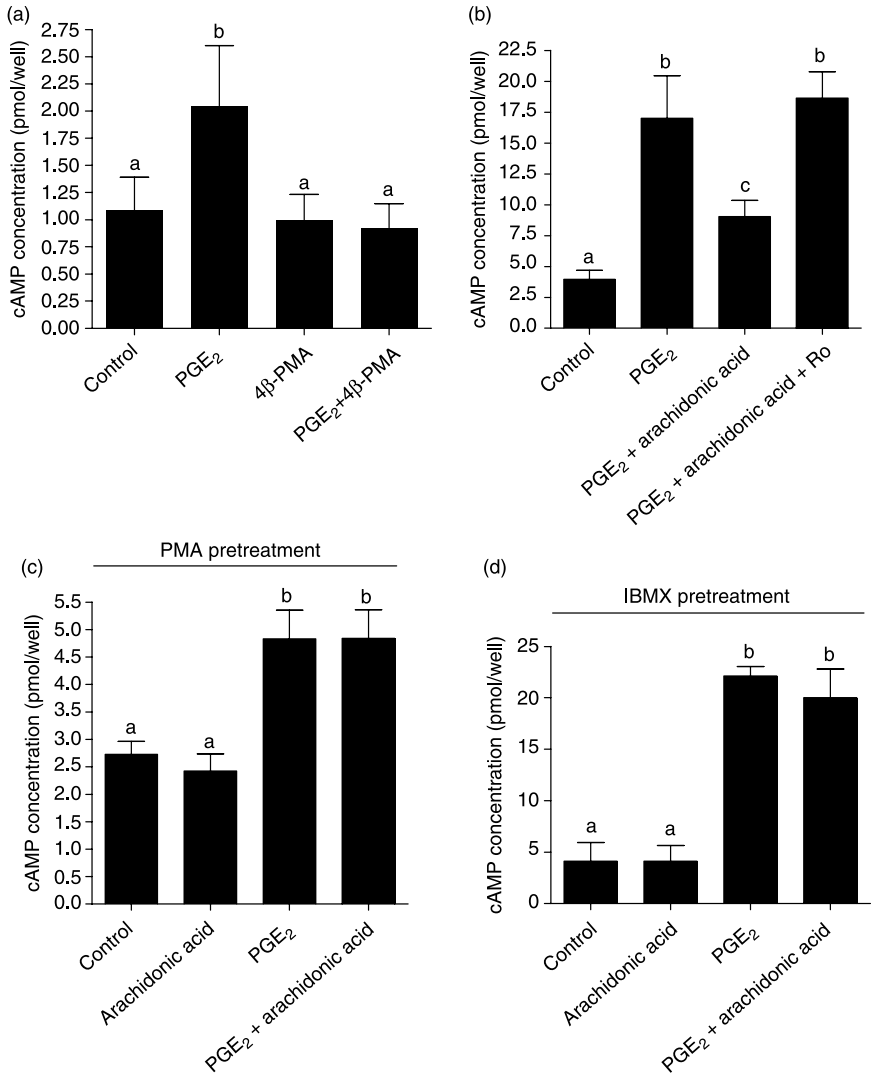

Figure 3 The inhibitory effect of arachidonic acid on PGE_2-induced cyclic AMP depends on protein kinase C (PKC) and phosphodiesterase. In each case, cultures were terminated 20 min after adding arachidonic acid or PGE_2. (a) PMA (2 μmol/l; PMA) added 10 min before PGE_2 prevented the rise in cyclic AMP concentration in response to 3 μmol/l PGE_2 ($P<0.01$). (b) The PKC inhibitor, RO318425 (500 nmol/l), added 10 min before arachidonic acid (50 μmol/l) and PGE_2 (3 μmol/l) prevented the antagonistic effect of arachidonic acid ($P<0.05$). (c) The inhibitory effect of arachidonic acid was absent in cells pre-treated with PMA for 24 h to downregulate PKC (compare with Fig. 2a). PGE_2 alone versus PGE_2 and arachidonic acid, $P>0.05$. (d) The inhibitory effect of arachidonic acid on PGE_2-induced cyclic AMP depended upon phosphodiesterase. Cells pre-treated for 30 min with the phosphodiesterase inhibitor IBMX (0.1 mmol/l) were subsequently treated with PGE_2 and/or arachidonic acid. Under these conditions, the inhibitory effect of arachidonic acid on PGE_2-induced cyclic AMP level was absent (compare with Fig. 2a). PGE_2 alone versus PGE_2 and arachidonic acid, $P>0.05$.

To confirm that the inhibitory effect of arachidonic acid on the cAMP pathway was reflected at the cell protein level, we measured concentrations of COX-2 in cells treated with the cell membrane-permeable cAMP analogue, dibutyryl cAMP. COX-2 is induced in the uterus by cAMP (Arosh *et al.* 2004) and is therefore an appropriate marker for effects exerted through this second messenger pathway. In these experiments, arachidonic acid blocked or reduced the effect of dibutyryl cAMP. This is consistent with the activation of PDE since dibutyryl cAMP is sequentially hydrolysed, once inside the cell, to monobutyryl cAMP and cAMP. The cAMP generated in this way then activates PKA. Therefore, increased cAMP-PDE activity would be expected to prevent PKA activation through removal of cAMP derived from dibutyryl cAMP and, hence, to block COX-2 accumulation. Identical effects were observed with PGE_2 (Fig. 4c) and forskolin, both of which activate adenylyl cyclase.

It was not our aim to identify the mechanisms by which COX-2 levels were affected, but to use COX-2 as an indicator of a cellular response, and therefore, we cannot differentiate between increased gene expression and reduced turnover of COX-2 transcripts or protein. However, COX-2 is under the transcriptional control of CREB through a well-recognized cAMP response element in the gene promoter (Zhou *et al.* 1999, Wu & Wiltbank 2002, Schroer *et al.* 2002). Therefore, it is probable that the increased COX-2 level in response to dibutyryl cAMP reflected increased transcription.

It is unlikely that the effect observed here is specific to arachidonic acid, as PKC is activated by a wide range of polyunsaturated fatty acids (Shinomura *et al.* 1991, Khan *et al.* 1995). In practice, the fatty acids involved will probably reflect the composition of the phospholipids from which they are derived. As shown by Elmes *et al.* (2004), arachidonic acid is present at a higher concentration than any other polyunsaturated fatty acid (PUFA)

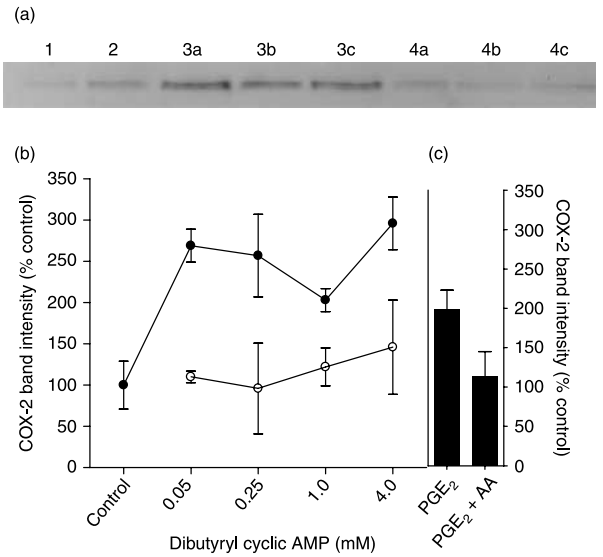

Figure 4 COX-2 protein levels determined by immunoblotting in bovine endometrial stromal cells. (a) Representative immunoblot showing: 1, untreated cells; 2, cells treated with arachidonic acid (50 μmol/l); 3a–c, separate cultures treated with dibutyryl cyclic AMP (0.25 μmol/l); 4a–c, separate cultures treated with dibutyryl cyclic AMP (0.25 μmol/l) and arachidonic acid (50 μmol/l). All treatments were for 6 h. All bands are from adjacent wells on a single gel. (b) Effects of dibutyryl cyclic AMP on COX-2 levels. BST cells were cultured for 6 h with dibutyryl cyclic AMP (0.05, 0.25, 1.0 and 4.0 mmol/l) with (open symbols) or without (closed symbols) arachidonic acid (50 μmol/l). Dibutyryl cyclic AMP increased COX-2 levels in BST cells (ANOVA; $P<0.001$). The effect of dibutyryl cyclic AMP was antagonized by arachidonic acid ($P<0.001$) at all dibutyryl cyclic AMP concentrations tested. (c) Arachidonic acid (50 μmol/l) antagonized the effect of PGE_2 (3 μmol/l) on COX-2 levels in cells cultured for 6 h. COX-2 level was increased by PGE_2 in the absence of arachidonic acid ($P<0.05$).

in phosphatidylcholine and phosphatidylethanolamine in the endometrium in ewes, and it is likely that the same applies in cattle. All the other six unsaturated fatty acids present are effective activators of PKC.

Endogenous synthesis of PGE_2 by the cells was unlikely to be a factor in the present investigation, as the culture media were changed immediately before test substances were added, hence removing prostanoids accumulated before the experimental period. Rates of production of PGE_2 by BST cells in the presence of 50 μmol/l arachidonic acid were ∼20 pmol/mg protein per min (ELR Sheldrick unpublished observations), which with 0.15 mg cell protein per well would be expected to produce a concentration of 6 nmol/l in 5 ml medium after 20 min incubation. This is ∼0.2% of the concentration of PGE_2 used to activate adenylyl cyclase (3 μmol/l), and would not be reached until the end of the 20 min incubation with test substances. Conversion of exogenous arachidonic acid to PGE_2 clearly did not prevent the inhibitory effect of arachidonic acid, presumably because culture times after addition of test substances were short.

A similar argument suggests that the effect of arachidonic acid was not likely to have resulted from the conversion to $PGF_{2\alpha}$. The rate of synthesis of $PGF_{2\alpha}$ in the presence of arachidonic acid was ∼1/100 that of PGE_2, giving a concentration of 0.06 nmol/l after 20 min. Although the stromal cells used here express the FP receptor (ELR Sheldrick unpublished observations), this concentration of $PGF_{2\alpha}$ is 0.006% of that required to activate phospholipase C in other cell types (Abayasekara *et al.* 1993).

In addition to their effects on PDE, arachidonic acid and other polyunsaturated fatty acids have both activatory and inhibitory actions on adenylyl cyclase, depending upon the adenylyl cyclase isoform and G proteins expressed in target cells. For instance in cells expressing the G protein G_z (a G_i isoform), such as neuronal cells and platelets, arachidonic acid inactivates G_z, leading to adenylyl cyclase activation (Glick *et al.* 1996). In contrast, in brain cell membrane preparations, arachidonic acid inhibits adenylyl cyclase by a direct interaction with the catalytic subunit (Nakamura *et al.* 2001). Arachidonic acid may also affect adenylyl cyclase activity through phosphorylation via PKC; in cells expressing type V adenylyl cyclase, phosphorylation by PKC is activatory (Kawabe *et al.* 1994), whereas in cells expressing type VI adenylyl cyclase, it is inhibitory (Lin *et al.* 2002). Therefore, the possibility exists that PUFA cause other actions through modulation of the cAMP/protein kinase pathway. Effects such as these did not appear to be important in the present experiments, where there was no change in basal cAMP concentration with arachidonic acid alone. However, the cells were exposed to arachidonic acid for a short time (20 min), and it is not possible to rule out an effect with a slower onset.

The concentration of arachidonic acid used in these experiments (50 μmol/l) was consistent with the concentration required to activate purified PKC (20–50 μmol/l; Shinomura *et al.* 1991). It is probably within the physiological range in the cells in terms of the intracellular level reached during the culture period. The concentration of free arachidonic acid in bovine stroma has not been reported, but can be inferred from the K_m of enzymes for which it is a substrate (for instance, COX-2, ∼5 μmol/l; Smith *et al.* 1996) and the concentration of arachidonic acid-containing phospholipids (∼800 nmol/g, i.e. 800 μmol/l on the basis of 1 ml/g; Elmes *et al.* 2004). On the other hand, the intracellular concentration of arachidonic acid is unlikely to have reached 50 μmol/l in the short time for which the cells were exposed to it (20 min), since arachidonic acid was added to medium containing charcoal-stripped serum, which would not prevent it gaining access to the cells, but might be expected to bind arachidonic acid avidly, reducing its availability.

In the endometrial stromal cells used here, arachidonic acid antagonized the stimulatory effect of dibutyryl cAMP or PGE_2 at the level of cell protein, as shown by measuring COX-2 levels (Fig. 4). This is

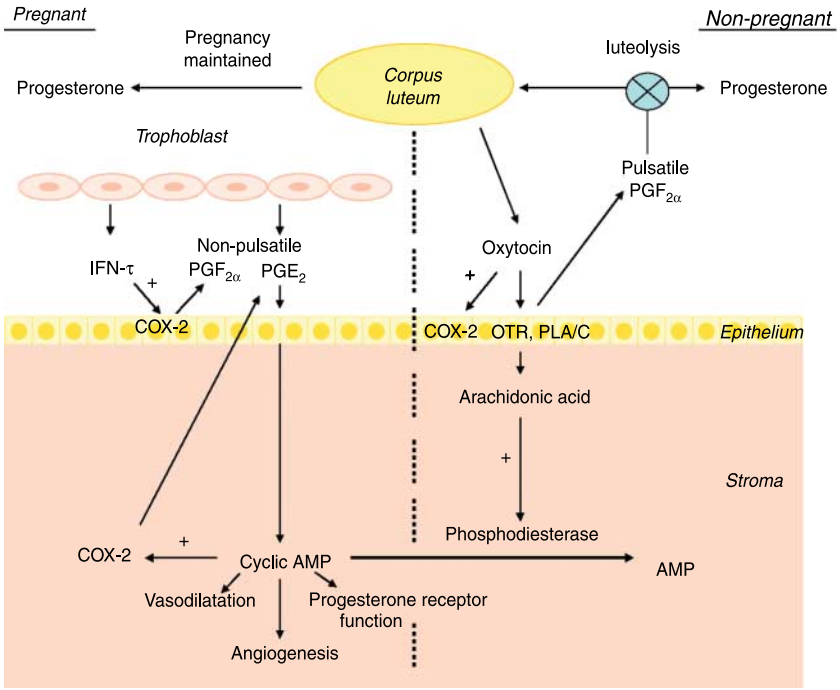

Figure 5 Proposed interactions between luteotrophic and luteolytic pathways in the choice between pregnancy and a return to cyclicity in polyoestrous ruminants, in which interferon-τ is the maternal recognition of pregnancy signal and there is no decidualization. Luteotrophic signals released by the pre-attachment conceptus include PGE_2 (secreted from day 10 post-conception) and interferon-τ (secreted from day 15). PGE_2 acts through EP2 receptors to activate adenylyl cyclase leading to raised levels of cyclic AMP and subsequently angiogenesis, vasodilatation, support for the progesterone receptor and induction of COX-2. The effect on COX-2, in turn, leads to further PGE_2 production through PGE synthase in the stroma. Interferon-τ (IFN-τ) prevents expression of the oxytocin receptor (OTR) in the epithelium and induces COX-2 in the epithelium. Absence of OTR prevents episodic $PGF_{2\alpha}$ secretion, and chronically elevated non-pulsatile $PGF_{2\alpha}$ and PGE_2 are antiluteolytic. In the absence of IFN-τ, this pathway is opposed by luteolytic mechanisms involving oxytocin secretion by the corpus luteum. Oxytocin acts through the oxytocin receptor expressed initially by epithelial cells to stimulate release of arachidonic acid through phospholipases A and C (PLA/C) and expression of COX-2. Arachidonic acid is converted to $PGF_{2\alpha}$ by the epithelium stimulating episodic secretion of oxytocin by the corpus luteum, leading to luteolysis. Arachidonic acid reduces cyclic AMP levels by activating a cyclic AMP-specific phosphodiesterase, thereby counteracting the effects of PGE_2 on the stroma.

consistent with the induction of PDE by arachidonic acid and confirms that the effect on cAMP level was reflected in protein synthesis. The results also show that arachidonic acid has both stimulatory and inhibitory effects on COX-2, since it increases COX-2 levels in bovine endometrial epithelial cells when added alone (Parent *et al.* 2003). These effects have different time courses, in that the action through PDE shown here is rapid (within 10 min), while the rise in COX-2 demonstrated by Parent *et al.* (2003) peaks at 6 h. The explanation for these apparently contradictory responses may lie in the promiscuity of the COX-2 promoter, which is sensitive to many transcription factors. Their reconciliation at a molecular level must await further understanding of the crosstalk between the second messenger pathways involved.

Events leading to PG production in bovine endometrial tissues are temporally and spatially separated. Beginning at about day 10 after conception, the conceptus secretes PGE_2, which initiates a positive feedback loop in the stroma whereby cAMP production leads to COX-2 induction and further production of PGE_2. After day 15, in the event that conceptus interferon-τ (IFN-τ) secretion is absent or insufficient, the OTR is expressed in the epithelium, leading to episodes of $PGF_{2\alpha}$ secretion, which result in luteolysis and a further chance to ovulate. Spatial separation arises because the epithelium expresses the OTR earlier than the stroma and is the principal source of $PGF_{2\alpha}$, whereas the stroma does not express the OTR until oestrus, and produces principally PGE_2 (Asselin *et al.* 1996, Robinson *et al.* 1999). Expression of COX-2 in the epithelium is increased both by oxytocin in the non-pregnant animal (Parent *et al.* 2003) and by IFN-τ in pregnancy (Emond *et al.* 2004). Interferons also activate phospholipase A_2 (Hannigan & Williams 1991), which would be expected to increase the availability of arachidonic acid to COX-2. In both cases, these effects lead to increased secretion of $PGF_{2\alpha}$. In the non-pregnant animal, the secretion of $PGF_{2\alpha}$ is pulsatile, as a result of a positive feedback loop stimulating further luteal secretion of oxytocin (Flint & Sheldrick 1982). In pregnancy, IFN-τ leads to increased secretion of $PGF_{2\alpha}$ (Peterson *et al.* 1976, Payne & Lamming 1994), but without pulses. Secretion of $PGF_{2\alpha}$ is required to be pulsatile in order to cause luteolysis, and as a result, oxytocin causes luteolysis but

IFN-τ does not. Indeed, a prolonged and sustained rise in $PGF_{2\alpha}$ in response to IFN-τ may lead to luteal refractoriness through downregulation of PKC (Abayasekara *et al.* 1993), and hence act as an antiluteolysin.

The physiological significance of the inhibitory action of arachidonic acid on the adenylyl cyclase pathway presumably resides in inhibitory effects not only on COX-2 and the response to PGE_2, but also on other compounds acting through cAMP. The effect of arachidonic acid on PGE_2-induced cAMP accumulation observed here would be expected to prevent responses to agents elevating cAMP levels such as relaxin, activin, luteinizing hormone and corticotrophin-releasing hormone (Bartscha & Ivell 2004, Tierney & Giudice 2004). In this context, arachidonic acid may be viewed as a component of a complex of luteolytic signals maintaining the endometrium in a non-pregnant state characterized by a low level of cAMP, absence of decidualization, vasodilatation and endometrial angiogenesis, and lack of support for the progesterone receptor (Gellerson & Brosens 2003). These interactions are summarized for ruminants in Fig. 5. The outcome of a reduction in endometrial cAMP level may therefore be to ensure blockade of signals associated with pregnancy, such as increased stromal COX-2 and PGE_2 production, in the event IFN-τ production is low and pregnancy is likely to fail. This would impart the selection advantage of securing resumption of cyclicity and a further chance of conception at the earliest opportunity.

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