Prostaglandin moieties that determine receptor binding specificity in the bovine corpus luteum

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This study provided a pharmacological evaluation of prostaglandin binding to bovine luteal plasma membrane. It was found that [³H]PGF_{2α}, [³H]PGE₂, [³H]PGE₁ and [³H]PGD₂ all bound with high affinity to luteal plasma membrane but had different specificities. Binding of [³H]PGF_{2α} and [³H]PGD₂ was inhibited by non-radioactive PGF_{2α} (IC₅₀ values of 21 and 9 nmol l⁻¹, respectively), PGD₂ (35 and 21 nmol l⁻¹), and PGE₂ (223 and 81 nmol l⁻¹), but not by PGE₁ (> 10 000 and 5616 nmol l⁻¹). In contrast, [³H]PGE₁ was inhibited by non-radioactive PGE₁ (14 nmol l⁻¹) and PGE₂ (7 nmol l⁻¹), but minimally by PGD₂ (2316 nmol l⁻¹) and PGF_{2α} (595 nmol l⁻¹). Binding of [³H]PGE₂ was inhibited by all four prostaglandins, but slopes of the dissociation curves indicated two binding sites. Binding of [³H]PGE₁ was inhibited, resulting in low IC₅₀ values, by pharmacological agonists that are specific for EP₃ receptor and possibly EP₂ receptor. High affinity binding of [³H]PGF_{2α} required a C15 hydroxyl group and a C1 carboxylic acid that are present on all physiological prostaglandins. Specificity of binding for the FP receptor depended on the C9 hydroxyl group and the C5/C6 double bond. Alteration of the C11 position had little effect on affinity for the FP receptor. In conclusion, there is a luteal EP receptor with high affinity for PGE₁, PGE₂, agonists of EP₃ receptors, and some agonists of EP₂ receptors. The luteal FP receptor binds PGF_{2α}, PGD₂ (high affinity), and PGE₂ (moderate affinity) but not PGE₁ due to affinity determination by the C9 and C5/C6 moieties, but not the C11 moiety.

Introduction

Prostaglandins have a variety of regulatory functions in the corpus luteum. It is well established that PGF_{2α} is the primary initiator of luteolysis in many species, including all species of ruminant investigated (reviewed by Knickerbocker *et al.*, 1988). The physiological role of type E prostaglandins is not clearly defined, but PGE₂ or PGE₁ have been implicated in regulation of the duration of the oestrous cycle, luteal progesterone production, luteolysis, and maternal recognition of pregnancy (Kimball and Lauderdale, 1975; Gimenez and Henricks, 1983).

Cloning studies show that prostaglandin receptors are members of the seven transmembrane domain receptor superfamily (reviewed by Negishi *et al.*, 1995). Although prostaglandin receptors are similar in amino acid sequence and structure (Negishi *et al.*, 1995) and prostaglandins are similar in chemical structure, it has been reported that prostaglandins only bind and activate specific receptors (Coleman *et al.*, 1994). The current nomenclature for prostaglandin receptors corresponds to the naturally occurring prostanoid that binds to that receptor with the

highest affinity (for example PGD₂ binds to the DP receptor, PGF_{2α} binds to the FP receptor; Coleman *et al.*, 1995). There are multiple receptor subtypes that bind PGE₂ (EP₁, EP₂, EP₃, EP₄; reviewed by Ushikubi *et al.*, 1995) and specific pharmacological agonists have been used to differentiate EP receptor subtypes. Although a number of studies have evaluated prostaglandin binding in the corpus luteum of various species, no study has used specific pharmacological agonists to investigate the PGE receptor subtype in the corpus luteum. Accordingly, the first two experiments in this study were designed to characterize the specificity of prostaglandin binding and to identify the EP receptor subtypes in the bovine corpus luteum. Experiment 3 was designed to provide novel information on the precise moieties on the PGF_{2α} molecule that determine specificity of binding to the FP receptor.

Materials and Methods

Reagents

Misoprostol and SC-19220 were gifts from G. D. Searle (Skokie, IL). Sulprostone and M&B 28,767 were donated by Schering (Berlin) and Rhône-Poulenc Rorer Ltd (Dagenham),

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respectively. All other non-radioactive prostaglandins and prostaglandin derivatives were obtained from Cayman Chemical (Ann Arbor, MI). [^3H]PGD₂, [^3H]PGE₁, [^3H]PGE₂, and [^3H]PGF_{2 α} were purchased from New England Nuclear/Dupont (Boston, MA). Gelatin was purchased from Difco Laboratories (Detroit, MI). All other chemicals were obtained from Sigma Chemical Company (St Louis, MO).

Tissue homogenization

Mid-cycle corpora lutea were selected from bovine ovaries obtained from an abattoir. Minced corpora lutea were suspended in tissue homogenization buffer (10 mmol trizma hydrochloride l⁻¹, 20 mmol sucrose l⁻¹, 1 mmol CaCl₂ l⁻¹, 1 mmol MgCl₂ l⁻¹, 0.02% (w/v) NaN₃, pH 7.0). Tissue was homogenized on ice using an ultraturrax homogenizer (Tekmar Company, Cincinnati, OH) initially and then with a Wheaton glass tissue grinder (Fisher Scientific, Pittsburgh, PA). The plasma membrane fraction was obtained by centrifuging the homogenized tissues three times at 3000 g for 5 min at 4°C to remove tissue debris. The supernatant was then centrifuged at 30000 g for 30 min at 4°C. The plasma membrane pellet was resuspended in tissue homogenization buffer. Protein content was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots were stored at -70°C. Immediately before receptor binding studies, the tissue was thawed on ice and then centrifuged for 30 min at 30000 g. The pellet was resuspended in assay buffer (20 mmol 2-[N-morpholino]-ethanesulfonic acid (MES) l⁻¹, 10 mmol CaCl₂ l⁻¹, 10 mmol MgCl₂ l⁻¹, 0.02% (w/v) NaN₃, 0.1% (w/v) gelatin, pH 6.0). A single luteal plasma membrane preparation was used for Expts 1 and 2 and a different preparation was used for Expt 3.

Radioreceptor assays

A time and temperature course of the binding assay described below was run initially to determine a single time point and temperature at which [^3H]PGD₂, [^3H]PGE₁, [^3H]PGE₂ and [^3H]PGF_{2 α} showed stable binding. Three incubation temperatures (4°C, 20°C, and 37°C) and seven time points (0.5, 1, 2, 4, 8, 12 and 24 h) were examined. Binding was stable for all four [^3H]prostaglandins at 4°C between 12 and 24 h. All other radioreceptor assays were run at 4°C for 20 h.

Assays were conducted in low-binding 96-well microtitre plates (Fisher Scientific, Pittsburgh, PA) in 100 μl volume using 200 μg plasma membrane protein. Tritiated prostaglandins (3 nmol l⁻¹) were inhibited by various concentrations of non-radioactive prostaglandins or prostaglandin agonists (1–10000 nmol l⁻¹) diluted in assay buffer. Samples were incubated on a microtitre plate shaker (Fisher Scientific, Pittsburgh, PA) for 20 h at 4°C. Free prostaglandins were separated from bound prostaglandins by rapid filtration with a cell harvester. Plasma membrane protein (containing bound prostaglandin) was collected on glass fibre filter paper (Schleicher & Schuell Inc., Keene, NH) and washed thoroughly with wash buffer (20 mmol MES l⁻¹, 1 mmol CaCl₂ l⁻¹, 1 mmol MgCl₂ l⁻¹, 0.02% (w/v) NaN₃, pH 6.0) at 4°C.

Samples were placed in BioSafe II scintillation fluid (Beckman Co., Fullerton, CA), vortexed for 1 min, and stored in a dark area for at least 12 h. Samples were placed in a scintillation counter for 3 min to determine the amount of radioactivity. Inhibition curves were constructed for each radioactive and each non-radioactive prostaglandin.

Scatchard analysis was used to determine the number and affinity of FP and EP receptors by displacement of [^3H]PGE₁ and [^3H]PGF_{2 α} by non-radioactive PGE₁ and PGF_{2 α} , respectively (Scatchard, 1949). The procedures were similar to those for the inhibition curve assays. The assays were conducted in 96-well microtitre plates in 100 μl using 106.5 and 213.0 μg plasma membrane protein (15 and 30 mg initial weight of corpora lutea, respectively). [^3H]PGE₁ and [^3H]PGF_{2 α} (3 nmol l⁻¹) binding was inhibited by increasing amounts (0.06–6 nmol l⁻¹) of cold PGE₁ and PGF_{2 α} , respectively.

Non-radioactive PGD₂, PGE₁, PGE₂, or PGF_{2 α} (10 μmol l⁻¹ approximately equivalent to 3.5 μg prostaglandin ml⁻¹) was incubated for 20 h (4°C) with bovine luteal plasma membrane in conditions similar to those for the radioreceptor assay to test for degradation of PGF_{2 α} or conversion of prostaglandins to PGF_{2 α} . Plasma membrane was removed by centrifugation at 30000 g for 30 min at 4°C, and the assay buffer from each sample was analysed for PGF_{2 α} by radioimmunoassay (Townson and Pate, 1994).

Statistical analysis

The data from the inhibition curves in Expt 1 were applied to a logistic model to estimate the concentration at which 50% binding occurred and the slope of the curve at that point. The non-linear regression routine in S-plus software (Mathsoft Inc., Seattle, WA) was used. The model assumes that the response will start at 100% for low concentrations of non-radioactive prostaglandins and decrease toward 0% as the concentration of non-radioactive prostaglandin increases. The following form of the model was used:

$$p = 1 - \{1 / [1 + \exp((x - \theta_1) / \theta_2)]\}$$

where $x = \log_{10}$ (concentration of non-radioactive prostaglandin) and p = percentage [^3H]prostaglandin bound/100. With this parameterization, θ_1 is the x value on the log scale where the curve reaches half its height ($p = 0.5$ or the IC₅₀ value), and θ_2 is a scale parameter, measured in the units of the x -axis, and is related to the steepness of the curve. The derivative of the curve (slope) at 50% is $1/(4 \times \theta_2)$. The absolute value of θ_2 is the distance on the x -axis over which the response decreases from 73.1 to 50%, and from 50 to 26.9%, due to the symmetry of the curve. This parameter is inversely related to the slope of the curve; a large θ_2 reflects a relatively flat curve, whereas a small θ_2 corresponds to a steep rapidly descending curve. Within each curve, the variability appeared homogeneous. Among the curves there were differences in variability as reflected in the widths of the confidence intervals for the parameter estimates. Nominal 95% confidence intervals for θ_1 and θ_2 were computed using each parameter estimate and its standard error (Tables 1 and 2) to facilitate comparisons across multiple curves.

Table 1. Inhibition (50%) of radioactive prostaglandins by non-radioactive prostaglandins bound to bovine luteal plasma membrane

Radioactive prostaglandin	PGD ₂	PGE ₁	PGE ₂	PGF _{2α}
[³ H]PGD ₂	21 (18, 24)	5616 (4799, 6572)	81 (68, 97)	9 (8, 11)
[³ H]PGE ₁	2316 (1703, 3148)	14 (11, 17)	7 (6, 9)	595 (453, 782)
[³ H]PGE ₂	242 (172, 339)	81 (58, 112)	9 (7, 12)	66 (49, 88)
[³ H]PGF _{2α}	35 (27, 45)	> 10 000	223 (176, 282)	21 (17, 26)

$n = 3$ experiments performed in triplicate. IC₅₀ (nmol l⁻¹) = mean. Confidence intervals (95%) are shown in parentheses (lower limit, upper limit). For calculation of θ , and standard errors the data were in log units. Values were transformed to the inverse log of θ , to provide IC₅₀ values, thus the confidence intervals appear asymmetrical.

Table 2. Scale parameter (θ_2 = distance on x -axis from 73.1 to 50% bound) for displacement by non-radioactive prostaglandins of radioactive prostaglandins bound to bovine luteal plasma membrane*

Radioactive prostaglandin	PGD ₂	PGE ₁	PGE ₂	PGF _{2α}	Row average
[³ H]PGD ₂	0.48 (0.42, 0.54)	0.46 (0.39, 0.53)	0.60 (0.53, 0.66)	0.50 (0.44, 0.56)	0.51
[³ H]PGE ₁	0.74 (0.60, 0.88)	0.43 (0.33, 0.52)	0.49 (0.39, 0.58)	0.65 (0.54, 0.75)	0.57
[³ H]PGE ₂	0.99 (0.85, 1.13)	0.92 (0.79, 1.06)	0.60 (0.50, 0.70)	0.76 (0.65, 0.88)	0.82
[³ H]PGF _{2α}	0.53 (0.44, 0.62)	0.36 (0.19, 0.53)	0.46 (0.38, 0.55)	0.37 (0.30, 0.45)	0.43

*The curve is symmetrical so the scale parameter describes the slope between 26.9 and 73.1%. The estimated slope of the curve at 50% is $1/(4 \times \theta_2)$.

$n = 3$ experiments performed in triplicate. Values are predicted scale parameters from logistic model fit. Confidence intervals (95%) are shown in parentheses (lower limit, upper limit).

Results

Time and temperature effects on binding

All four tritiated prostaglandins demonstrated stable binding at 4°C between 12 and 24 h. This was not necessarily the highest point of binding for each individual prostaglandin. However, under these conditions, all [³H]prostaglandins bound with high affinity to bovine luteal plasma membrane.

Experiment 1: prostaglandin receptors in the corpus luteum

The number of [³H]PGF_{2α} molecules specifically bound to 200 µg plasma membrane protein was $6.7 \pm 0.5 \times 10^{10}$ (mean $\pm$ SEM). Binding of [³H]PGF_{2α} (Fig. 1a, Table 1) was inhibited by non-radioactive PGF_{2α} and PGD₂ with similar affinity (IC₅₀ = 21 and 35 nmol l⁻¹, respectively). Cold PGE₂ displaced [³H]PGF_{2α} binding with lower affinity (IC₅₀ = 223 nmol l⁻¹), whereas PGE₁ did not inhibit the binding of [³H]PGF_{2α} to luteal plasma membrane (IC₅₀ > 10 µmol l⁻¹).

The number of [³H]PGD₂ molecules specifically bound to 200 µg plasma membrane protein was $4.6 \pm 0.6 \times 10^{10}$ (mean $\pm$ SEM). Similar to [³H]PGF_{2α} binding, [³H]PGD₂ binding

(Fig. 1b, Table 1) was inhibited by PGD₂ (IC₅₀ = 21 nmol l⁻¹), PGF_{2α} (IC₅₀ = 9 nmol l⁻¹) and PGE₂ (IC₅₀ = 81 nmol l⁻¹). Non-radioactive PGE₁ only displaced [³H]PGD₂ at a high concentration (IC₅₀ = 5616 nmol l⁻¹). The number of [³H]PGE₁ molecules specifically bound to 200 µg plasma membrane protein was $1.6 \pm 0.1 \times 10^{10}$ (mean $\pm$ SEM). Both PGE₁ and PGE₂ displaced the binding of [³H]PGE₁ to luteal plasma membrane with similar affinity (IC₅₀ = 14 and 7 nmol l⁻¹, respectively). At much higher concentrations, PGF_{2α} (IC₅₀ = 595 nmol l⁻¹) and PGD₂ (IC₅₀ = 2316 nmol l⁻¹) inhibited [³H]PGE₁ binding (Table 1, Fig. 1c).

The number of [³H]PGE₂ molecules specifically bound to 200 µg plasma membrane protein was $2.9 \pm 0.2 \times 10^{10}$ (mean $\pm$ SEM). Binding of [³H]PGE₂ was inhibited with a low concentration of PGE₂ (IC₅₀ = 9 nmol l⁻¹). Higher concentrations of PGF_{2α} (IC₅₀ = 66 nmol l⁻¹), PGE₁ (IC₅₀ = 81 nmol l⁻¹) and PGD₂ (IC₅₀ = 242 nmol l⁻¹) were required to displace [³H]PGE₂ (Fig. 1d, Table 1).

The scale parameters relate to the slope between 27% and 73% (Table 2) and were greatest for displacement of [³H]PGE₂. This is consistent with the relatively flat displacement lines for [³H]PGE₂ (Fig. 1d). Two other scale parameters that were relatively large were for the displacement of [³H]PGE₁ by PGD₂ and PGF_{2α}. The remaining scale parameters were between 0.36 and 0.60. From these results, the decision was

Fig. 1. Inhibition of (a) $[^3\text{H}]\text{PGF}_{2\alpha}$, (b) $[^3\text{H}]\text{PGD}_2$, (c) $[^3\text{H}]\text{PGE}_1$ and (d) $[^3\text{H}]\text{PGE}_2$ by various non-radioactive prostaglandins in bovine luteal plasma membrane. $n = 3$ experiments in triplicate.

made to use $[^3\text{H}]\text{PGE}_1$ to characterize further the EP receptors, since the $[^3\text{H}]\text{PGE}_2$ binding results were compromised by the apparent binding of PGE_2 to both EP and FP receptors.

The radioimmunoassay did not detect an increase in the concentration of $\text{PGF}_{2\alpha}$ in the assay buffer after 20 h incubation of high concentrations of non-radioactive PGD_2 , PGE_1 , PGE_2 or $\text{PGF}_{2\alpha}$ with bovine luteal plasma membrane, indicating that PGD_2 , PGE_1 and PGE_2 were not being converted to $\text{PGF}_{2\alpha}$ (data not shown). There appeared to be some crossreactivity of PGD_2 and PGE_2 with the $\text{PGF}_{2\alpha}$ antibody, since a value of 0.1–1.0 ng $\text{PGF}_{2\alpha} \text{ ml}^{-1}$ was detected for PGD_2 and PGE_2 before incubation with plasma membrane. The $\text{PGF}_{2\alpha}$ concentration detected in the assay buffer before and after 20 h incubation of PGD_2 or PGE_2 with bovine luteal plasma membrane decreased by 60% and 97%, respectively, indicating possible prostaglandin degradation.

Scatchard analysis showed a single high affinity binding site for $[^3\text{H}]\text{PGE}_1$ and $[^3\text{H}]\text{PGF}_{2\alpha}$ (Fig. 2). The dissociation constants (K_d) for the EP receptor were 7.6 and 9.2 nmol l^{-1} for 106.7 and 213.0 μg luteal plasma membrane protein, respectively (equivalent to 15 and 30 mg of initial corpus luteum). The concentration of EP receptors (B_{max}) was 437 and 507 amol μg^{-1} protein for 213.0 and 106.7 μg plasma membrane protein, respectively (Fig. 2). The dissociation constant for the FP receptor was 11.4 nmol l^{-1} for 216 μg plasma membrane protein and 11.1 nmol l^{-1} for 106.7 μg plasma membrane protein. The concentration of FP receptors in the plasma membrane was 2915 and 2986 amol μg^{-1}

protein for 213.0 and 106.5 μg plasma membrane protein, respectively (Fig. 2).

Experiment 2: EP receptor subtype determination

A variety of relatively specific agonists was used to inhibit $[^3\text{H}]\text{PGE}_1$ binding to determine the EP receptor subtype in the bovine corpus luteum. M&B 28,767 (EP_3 receptor agonist), 11-deoxy PGE_1 ($\text{EP}_2 > \text{EP}_3$), 16,16-dimethyl PGE_2 ($\text{EP}_3 > \text{EP}_1 > \text{EP}_2$), 17-phenyl trinor PGE_2 ($\text{EP}_1 > \text{EP}_3$), and sulprostone ($\text{EP}_3 > \text{EP}_1$) displaced $[^3\text{H}]\text{PGE}_1$ binding with relatively high affinity ($\text{IC}_{50} = 3, 8, 9, 17$ and 23 nmol l^{-1} , respectively) (Table 3). Misoprostol ($\text{EP}_2 > \text{EP}_3$) inhibited $[^3\text{H}]\text{PGE}_1$ binding at a higher concentration ($\text{IC}_{50} = 82 \text{ nmol l}^{-1}$), whereas SC-19220 (EP_1) did not displace $[^3\text{H}]\text{PGE}_1$ binding ($\text{IC}_{50} > 10000 \text{ nmol l}^{-1}$) (Table 3). Similar to $\text{PGF}_{2\alpha}$, fluprostenol (FP receptor agonist) was not able to inhibit $[^3\text{H}]\text{PGE}_1$ binding effectively ($\text{IC}_{50} = 6167 \text{ nmol l}^{-1}$).

Experiment 3: determination of essential sites for $\text{PGF}_{2\alpha}$ binding to FP receptor

Alterations to C1 of $\text{PGF}_{2\alpha}$ markedly decreased the ability of prostaglandin derivatives to inhibit $[^3\text{H}]\text{PGF}_{2\alpha}$ binding. The IC_{50} value for $\text{PGF}_{2\alpha}$ dimethyl amide was $> 10 \mu\text{mol l}^{-1}$ and for $\text{PGF}_{2\alpha}$ isopropyl ester was $383 \pm 27 \text{ nmol l}^{-1}$ (Table 4).

Fig. 2. Scatchard plot of the binding of (a) $[^3\text{H}]\text{PGE}_1$ (213 μg luteal plasma membrane protein: $K_d = 7.6 \pm 0.3 \text{ nmol l}^{-1}$; $B_{\text{max}} = 437 \pm 14 \text{ amol } \mu\text{g}^{-1}$ plasma membrane protein; 106.7 μg luteal plasma membrane protein: $K_d = 9.2 \pm 0.3 \text{ nmol l}^{-1}$; $B_{\text{max}} = 507 \pm 28 \text{ amol } \mu\text{g}^{-1}$ plasma membrane protein) and (b) $[^3\text{H}]\text{PGF}_{2\alpha}$ (213 μg luteal plasma membrane protein: $K_d = 11.4 \pm 2.0 \text{ nmol l}^{-1}$; $B_{\text{max}} = 2915 \pm 225 \text{ amol } \mu\text{g}^{-1}$ plasma membrane protein; 106.7 μg luteal plasma membrane protein: $K_d = 11.1 \pm 1.5 \text{ nmol l}^{-1}$; $B_{\text{max}} = 2986 \pm 225 \text{ amol } \mu\text{g}^{-1}$ plasma membrane protein) to a pool of bovine luteal plasma membrane. ($\square, \blacksquare$) 213 μg luteal plasma membrane protein; ($\circ, \bullet$) 106.7 μg luteal plasma membrane protein.

Similarly, oxidation at C15 (15-keto $\text{PGF}_{2\alpha}$) substantially altered binding of $[^3\text{H}]\text{PGF}_{2\alpha}$ ($\text{IC}_{50} = 667 \pm 145 \text{ nmol l}^{-1}$) (Table 4).

Reduction at C9 (PGE_2) had a moderate effect on $[^3\text{H}]\text{PGF}_{2\alpha}$ binding ($\text{IC}_{50} = 157 \pm 27 \text{ nmol l}^{-1}$). Similarly, changes at the double bond between C5 and C6 decreased inhibition of $[^3\text{H}]\text{PGF}_{2\alpha}$ binding (5-*trans* $\text{PGF}_{2\alpha}$, $\text{PGF}_{1\alpha}$; $\text{IC}_{50} = 61 \pm 5$, 153 ± 22 , respectively; Table 4).

Changes at other sites in $\text{PGF}_{2\alpha}$ had a minimal effect on inhibition of $[^3\text{H}]\text{PGF}_{2\alpha}$ binding. PGD_2 and 11-deoxy $\text{PGF}_{2\alpha}$ (changes at C11) had IC_{50} values of 27 ± 3 and $37 \pm 4 \text{ nmol l}^{-1}$, respectively. Hydrogenating C13 and C14 produced a slight change in $[^3\text{H}]\text{PGF}_{2\alpha}$ inhibition (13,14-dihydro $\text{PGF}_{2\alpha}$; $\text{IC}_{50} = 21 \pm 5 \text{ nmol l}^{-1}$). Dehydration at C17 and C18 ($\text{PGF}_{3\alpha}$) resulted in an IC_{50} value of $39 \pm 5 \text{ nmol l}^{-1}$. Changes beyond C16 had little effect on binding ($\text{PGF}_{3\alpha}$, fluprostenol, cloprostenol; $\text{IC}_{50} = 39 \pm 5$, 34 ± 6 and $5 \pm 2 \text{ nmol l}^{-1}$, respectively; Table 4).

Discussion

This study provides a pharmacological analysis of prostaglandin binding in the bovine corpus luteum. The systematic approach used in these experiments provided insight into the nature of prostaglandin binding in the corpus luteum.

It is apparent that PGE_2 binds to both FP and EP receptors. This is indicated by the inhibition of $[^3\text{H}]\text{PGF}_{2\alpha}$ binding by PGE_2 and, conversely, $\text{PGF}_{2\alpha}$ inhibition of $[^3\text{H}]\text{PGE}_2$ binding. In addition, the flattened slopes for inhibition of $[^3\text{H}]\text{PGE}_2$ by PGD_2 , $\text{PGF}_{2\alpha}$ and PGE_1 indicated that there are two different binding sites with different affinities for $[^3\text{H}]\text{PGE}_2$. This is also supported by the observation that the number of $[^3\text{H}]\text{PGE}_2$ molecules (2.9×10^{10}) bound to the bovine luteal

plasma membrane was higher than the number of $[^3\text{H}]\text{PGE}_1$ (1.6×10^{10}) molecules bound. Therefore, studies in which PGE_2 is used for either binding or physiological evaluations must be considered at least partially compromised because of this crossreactivity. For example, treatment of ovine large luteal cells with PGE_2 caused an increase in free intracellular calcium (Wiepz *et al.*, 1993) with an ED_{50} value (168 nmol l^{-1}) that corresponds closely to the affinity of the FP receptor for PGE_2 observed in this study and in other investigations (Wiepz *et al.*, 1992).

In contrast, PGE_1 appeared to interact with only the EP receptor and not the FP receptor (this study; Rao, 1974; Kimball and Lauderdale, 1975; Wiepz *et al.*, 1992). This was demonstrated by the large IC_{50} values for inhibition of $[^3\text{H}]\text{PGD}_2$ or $[^3\text{H}]\text{PGF}_{2\alpha}$ by PGE_1 . Similarly, very high concentrations of PGD_2 and $\text{PGF}_{2\alpha}$ were required to inhibit binding of $[^3\text{H}]\text{PGE}_1$. Several studies have demonstrated that PGE_1 and PGE_2 can have very different physiological effects on the corpus luteum. For example, in ewes in which the ovarian pedicle was treated with $\text{PGF}_{2\alpha}$, there was a decrease in luteal weight and serum progesterone concentration that could be restored to values observed in vehicle-treated ewes by simultaneous administration of PGE_1 but not PGE_2 (Reynolds *et al.*, 1981). In addition, the luteal phase of ewes was extended by administration of PGE_1 into the uterine horn ipsilateral but not contralateral to the corpus luteum (Huie *et al.*, 1981). Likewise, administration of high doses of PGE_1 in pseudopregnant rats delayed luteolysis, whereas administration of high doses of PGE_2 accelerated the onset of luteolysis (Weems *et al.*, 1979). Therefore, researchers interested in studying actions mediated through EP receptors in the corpus luteum should consider using PGE_1 or other specific EP receptor agonists, rather than PGE_2 because of the interaction of PGE_2 with FP as well as EP

Table 4. Prostaglandin F_{2α} analogues, structure and inhibition constants

Prostaglandin	Chemical structure	IC ₅₀ (nmol l ⁻¹)	Differs from PGF _{2α} at
PGF _{2α}		10	—
PGF _{2α} dimethyl amide		> 10 000	C1
PGF _{2α} isopropyl ester		383	C1
5- <i>trans</i> PGF _{2α}		61	C5/C6 bond
PGF _{1α}		153	C5/C6 bond
PGE ₂		157	C9
PGD ₂		27	C11
11-deoxy PGF _{2α}		37	C11
13,14-dihydro PGF _{2α}		21	C13/C14 bond
15-keto PGF _{2α}		667	C15
PGF _{3α}		39	C17/C18 bond
Fluprostenol		34	C17 tail
Cloprostenol		5	C17 tail

Arrows indicate the sites at which the analogues differ from PGF_{2α}.

receptors. This may be less of a problem in tissues that contain small numbers of FP receptors (Tsai *et al.*, 1998). The obvious problem with using PGE₁ is that PGE₂ appears to be the physiological ligand for EP receptors in most cases. For example, the uterine concentrations of PGE₂ are approximately tenfold greater than those of PGE₁ (Wilson *et al.*, 1972). Thus, it is unclear whether crossreactivity of PGE₂ with the FP receptor is involved in normal physiological events or is a pharmacological phenomenon.

Although PGD₂ binds with high affinity to luteal plasma membrane, it is likely that PGD₂ is binding to the FP receptor rather than a specific DP receptor. In preliminary experiments, a specific agonist for the DP receptor (BW-245C) was unable to displace either [³H]PGD₂ or [³H]PGF_{2α} binding in bovine luteal plasma membrane (IC₅₀ > 10 μmol l⁻¹; L. E. Anderson and M. C. Wiltbank, unpublished). Wiepz *et al.* (1992) reported that PGD₂ inhibits [³H]PGF_{2α} binding in the ovine corpus luteum. Binding studies in mammalian COS cells transfected with human FP receptor also demonstrated that PGD₂ inhibits binding of [³H]PGF_{2α} (Abramovitz *et al.*, 1994). Cloning and expression of the bovine FP receptor also showed crossreactivity with PGD₂ (Sakamoto *et al.*, 1994). Higher concentrations of PGD₂ were required to displace [³H]PGF_{2α} binding to mouse and rat FP receptors (Lake *et al.*, 1994; Sugimoto *et al.*, 1994), indicating a species difference in receptor crossreactivity. Interestingly, the DP receptor does not appear to bind either PGE₂ or PGF_{2α} in mice or humans (Hirata *et al.*, 1994; Boie *et al.*, 1995).

Experiment 2 was designed to determine the subtype of luteal EP receptor using pharmacological agonists. The finding that M&B 28,767 displaced [³H]PGE₁ with high affinity implies that an EP₃ type receptor is present (Negishi *et al.*, 1993). In addition, 16,16-dimethyl PGE₂ preferentially binds EP₃ receptors, although this compound may have non-selective binding to all EP receptors in some species (Jumblatt and Paterson, 1991; Li *et al.*, 1993). Furthermore, high affinity binding of sulprostone indicates that most luteal prostaglandin E binding is due to EP₃ receptors (Watabe *et al.*, 1993; Yang *et al.*, 1994). The EP₂ receptor agonists available crossreact with the EP₃ receptor, thus the presence of an EP₂ receptor could not be confirmed. The high affinity binding of 11-deoxy PGE₁ (Regan *et al.*, 1994) indicates that an EP₂ receptor may be present. However, misoprostol bound with lower affinity, as may be expected for an EP₃ receptor. Although several EP receptor agonists bind to the EP₁ receptor, most of these also bind to EP₃ receptors. The lack of binding of the EP₁ selective agonist SC-19220 (Coleman *et al.*, 1985) indicates that EP₁ receptors may not be present in the bovine corpus luteum, although low solubility of this compound may cause aberrant results. The slope of the inhibition curves indicated that the affinity of only one receptor was being inhibited by each of the agonists. Thus, these results are consistent with the luteal prostaglandin E receptor being of the EP₃ receptor subtype. An EP₂ receptor may also be present, but could not be demonstrated directly. Further studies with EP receptor-specific antibodies would provide more direct evidence for these conclusions.

Experiment 3 provided insight into the moieties involved

in PGF_{2α} binding. The C9 position was the only moiety that altered PGF_{2α} binding, and was unique to the PGF_{2α} molecule compared with the other common prostaglandins. Changing the C9 from hydroxyl to ketone (PGF_{2α} to PGE₂) increased the IC₅₀ value by more than tenfold. In contrast, alteration of the other unique site for PGF_{2α}, C11, had a minimal effect on PGF_{2α} binding. This could be demonstrated by changing the C11 site to a ketone (PGD₂) or by complete removal of the hydroxyl group (11-deoxy PGF_{2α}). Interestingly, removal of the C11 hydroxyl from the PGE₂ molecule also had little effect on PGE₁ binding. Thus, it appears that C9 offers the primary site for luteal prostaglandin receptor specificity, whereas C11 moieties appear to have minimal effect on bovine luteal EP or FP receptor binding.

The three groups that were important for PGF_{2α} binding but were not specific to the PGF_{2α} molecule were C1, C5/C6, and C15. Replacement of the C1 carboxylic acid with large groups (dimethyl amide or isopropyl ester) almost eliminated binding to the receptor. Unfortunately, there were no compounds available that altered only the nature of the carboxylic acid without the addition of large residues. It has been proposed that the C1 carboxylic acid forms a Schiff base with conserved arginine in the seventh transmembrane domain (residue 291 of the bovine FP receptor; Sakamoto *et al.*, 1994) and stabilizes binding of all prostaglandins to their receptors.

The C5/C6 double bond is important for PGF_{2α} binding, as indicated by the finding that removal of this double bond (PGF_{1α}) increased the IC₅₀ value by more than tenfold, and isomerization of the bond from *cis* to *trans* conformation (5-*trans* PGF_{2α}) significantly increased the IC₅₀ value. Although this bond is not unique to the PGF_{2α} molecule, it appears to have a major impact on receptor specificity. This is demonstrated by the fact that PGE₂ (C5/C6 double bond intact) has a moderate IC₅₀ value (157 nmol l⁻¹), whereas PGE₁ (C5/C6 double bond removed) caused virtually no displacement of [³H]PGF_{2α} (> 10 000 nmol l⁻¹). The C5/C6 double bond did not alter luteal prostaglandin E binding, as shown by high affinity binding of PGE₁ or PGE₂ as well as a number of different agonists that contained (sulprostone, 16,16-dimethyl PGE₂, 17-phenyl trinor PGE₂) or did not contain (M&B 28,767, 11-deoxy PGE₁) the C5/C6 double bond.

The hydroxyl in the C15 position appears to be essential for binding because changing this group to a ketone (15-keto PGF_{2α}) markedly reduced affinity for the FP receptor. It is possible that the hydroxyl in the C15 position, along with the carboxylic acid at C1, provide ionic interactions that stabilize PGF_{2α} binding to the FP receptor. There was little effect on PGF_{2α} or prostaglandin E binding or activity when alterations were made in the molecule beyond C17, similar to the findings of other studies (Negishi *et al.*, 1993; Watabe *et al.*, 1993; Yang *et al.*, 1994; Graves *et al.*, 1995). The addition of larger groups, such as phenols, after C17 has been used to increase the half-life for prostaglandins *in vivo* since these compounds are less susceptible to 15-hydroxy prostaglandin dehydrogenase (Miller *et al.*, 1975; Granström, 1975). Phenyl-substituted PGF_{2α} is at least as potent as PGF_{2α} (Miller *et al.*, 1975; Resul *et al.*, 1993).

In summary, high affinity binding of prostaglandins to

bovine luteal plasma membrane indicated the presence of specific FP and EP receptors. The affinity of different prostaglandins and prostaglandin analogues for these receptors indicated moieties on the prostaglandin molecule that are essential for binding to luteal prostaglandin receptors and that luteal EP receptor is probably of the EP₃ subtype, although results on the presence of luteal EP₂ receptors are equivocal. The group attached to the C9 position appears to be most critical for determining specificity of luteal FP and EP receptors. A *cis* double bond at C5/C6 is also critical for luteal FP but not EP receptor binding. Other parts of the prostaglandin molecule, including C11, appear to be minimally important for determination of luteal prostaglandin receptor specificity.

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