Prostaglandin receptors and role of G protein-activated pathways on corpora lutea of pseudopregnant rabbit *in vitro*

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Abstract

Studies were conducted to characterize receptors for prostaglandin (PG) $F_{2\alpha}$ ($PGF_{2\alpha}$) and PGE_2, and the signalling pathways regulating total nitric oxide synthase activity and progesterone production in rabbit corpora lutea (CL) of different luteal stages. CL were obtained at days 4, 9 and 13 of pseudopregnancy and cultured *in vitro* for 2 h with $PGF_{2\alpha}$ or PGE_2 and with activators and inhibitors of G protein (Gp), phospholipase C (PLC), protein kinase C (PKC), adenylate cyclase (AC) and protein kinase A (PKA).

High affinity $PGF_{2\alpha}$ receptor ($K_d = 1{\cdot}9 \pm 0{\cdot}6$ nM mean ± S.E.M.) concentrations increased ($P \leq 0{\cdot}01$) four- to five-fold from early to mid- and late-luteal phases ($50{\cdot}6 \pm 8{\cdot}5$, $188{\cdot}3 \pm 36{\cdot}1$ and $231{\cdot}4 \pm 38{\cdot}8$ fmol/mg protein respectively). By contrast, PGE_2 receptor ($K_d = 1{\cdot}6 \pm 0{\cdot}5$ nM) concentrations decreased ($P \leq 0{\cdot}01$) from day 4 to day 9 and 13 ($27{\cdot}5 \pm 7{\cdot}7$, $12{\cdot}4 \pm 2{\cdot}4$ and $16{\cdot}5 \pm 3{\cdot}0$ fmol/mg protein respectively). The Gp-dependent AC/PKA pathway was triggered only on day 4 CL, mimicking the PGE_2 treatment and increasing progesterone production. In both day 9 and day 13 CL, the Gp-activated PLC/PKC pathway evoked a luteolytic effect similar to that induced by $PGF_{2\alpha}$. The time-dependent selective resistance to $PGF_{2\alpha}$ and PGE_2 by rabbit CL is mediated by factors other than a lack of luteal receptor–ligand interactions.

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Introduction

There is now considerable direct evidence, derived by both *in vivo* (Carlson & Gole 1978, Kehl & Carlson 1981, Marcinkiewicz *et al.* 1992) and *in vitro* studies (O'Grady *et al.* 1972), supporting the hypothesis that in the rabbit, as in several other animal species, the major luteolytic hormone is prostaglandin (PG) $F_{2\alpha}$ ($PGF_{2\alpha}$). However, while there is a general agreement upon the luteolytic role of $PGF_{2\alpha}$, there is still some controversy about the mechanisms that protect prostaglandin-induced regression of corpora lutea (CL) in both the early- and mid-luteal phase of pseudopregnancy (Marcinkiewicz *et al.* 1992, Boiti *et al.* 1998). By contrast, for a long time PGE_2 has been recognized as a luteotrophic prostaglandin, as it may protect the CL from many of the actions induced by $PGF_{2\alpha}$ (Zelinsky-Wooten & Stouffer 1990, Ford & Christenson 1991).

It is also widely accepted that the effects induced by PGs reflect the interactions on target cells with specific surface receptors, and probably with different receptor subtypes, which influence second messenger systems via G protein (Gp) activation. Therefore, the increasing responsiveness of CL to $PGF_{2\alpha}$, or its analogues, during subsequent luteal developmental stages (Boiti *et al.* 1998), from total to partial refractoriness and complete luteolysis, may be due to changes in the number of receptors for $PGF_{2\alpha}$ and/or PGE_2.

Recently we demonstrated, by *in vitro* studies on CL of pseudopregnant rabbits, that $PGF_{2\alpha}$ and PGE_2 affect progesterone release differently, depending on the luteal stage, by regulating the activity of nitric oxide synthase (NOS), the enzyme which controls nitric oxide (NO) production (Boiti *et al.* 2000). On day 4 CL, PGE_2 was found to depress NOS activity and increase progesterone production, but was totally ineffective on day 9 CL. Prostaglandin $F_{2\alpha}$ up-regulated NOS activity and induced functional luteolysis in day 9 CL, but had no effect on young CL collected at the early luteal phase.

Several studies have documented a good correlation between *in vitro* binding specificity and concentrations of prostaglandin receptors for $PGF_{2\alpha}$ in membranes prepared from CL of several species and its luteolytic activity *in vivo* (Rao 1974, Wright *et al.* 1980). Additional reports have shown that plasma membrane receptors for PGE_2 in luteal tissue and luteal cells are also related to the functional status of CL during the oestrous cycle and pregnancy (Wiepz *et al.* 1992, Feng & Almond 1996). The presence of $PGF_{2\alpha}$ and PGE_2 binding sites in both large and small luteal cells has been verified in cow (Alila *et al.* 1987,

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Chegini *et al.* 1991), sheep (Balapure *et al.* 1989) and monkey (Brannian & Stouffer 1991) resulting in some discrepancies probably reflecting species differences (Niswender *et al.* 1985). However, the specific binding sites for either $PGF_{2\alpha}$ or PGE_2 have not been investigated in the rabbit.

The present work was undertaken to examine the affinity and concentration of receptors for $PGF_{2\alpha}$ and PGE_2 on CL of rabbits in the early-, mid- and late-luteal phase at days 4, 9 and 13 of pseudopregnancy by radioligand binding. These stages of pseudopregnancy were selected because the corresponding CL showed different age-related responsiveness to $PGF_{2\alpha}$ *in vivo* (Carlson & Gole 1978, Marcinkiewicz *et al.* 1992, Boiti *et al.* 1998). Moreover, to better elucidate the differential age-related effects of PGs and the sites of their functional actions on progesterone release and total NOS activity, the signal transduction pathway was activated *in vitro* in a stepwise fashion at the level of the receptor (Gp), phospholipase C (PLC) and protein kinase C (PKC), adenylate cyclase (AC) and protein kinase A (PKA). Additionally, the regulatory role of Gps in both luteolytic and luteotrophic actions was studied by adding a number of inhibitors for PLC, PKC, AC and PKA together with a NOS inhibitor (L-NAME).

Materials and Methods

Materials

Medium 199 was obtained from Gibco (Grand Island, NY, USA). Hepes, $NaHCO_3$, BSA, $PGF_{2\alpha}$ and PGE_2 were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA). [1,2,6,7-^{3}H]Progesterone was purchased from Amersham Int. (Buckinghamshire, UK), while non-radioactive progesterone and progesterone antiserum came from Sigma. [^{3}H]$PGF_{2\alpha}$ (5, 6,8,9,11,12, 14,15-^{3}H(N)-$PGF_{2\alpha}$, 218 Ci/mmol, NET 433) and [^{3}H]PGE_2 (5,6,8,11,12,14,15-^{3}H(N)-PGE_2, 200 Ci/mmol, NET 428) were purchased from New England Nuclear (Boston, MA, USA). Tissue culture plates with 24 flat bottom wells were obtained from Becton Dickinson & Co. (Clifton, NJ, USA). Bio-Rad Protein Assay kit was obtained from Bio-Rad Lab (Hercules, CA, USA). The following activators were purchased from Calbiochem Corp. (San Diego, CA, USA): Gp, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγ-S); PLC, imipramine; PKC, phorbol-12-myristate-13-acetate (PMA); AC, forskolin, 7-deacetyl-7-[*O*-(*N*-methylpiperaziono)-γ-butyryl] (L85 8051); PKA, adenosine 3′,5′-cyclic monophosphate, 8-bromo (8-bromo-cAMP). The following inhibitors for Gp, guanosine 5′-*O*-(2-thiodiphosphate), (GDPβ-S); PLC, ET-18-OCH_3; PKA, compound H89 were also purchased from Calbiochem, while those for PKC, staurosporine, AC, 2-*O*-methyladenosine, NOS, L-NAME came from Sigma. [2,3-^{3}H]L-Arginine, having a specific activity of 30–40 Ci/mmol, was purchased from Sigma. Scintillation fluid, Ultima Gold, was obtained from Packard (Groningen, The Netherlands). Multiscreen Assay System and FB Microplate Glass Fiber Type B Filter (MAFB N0B 10) were purchased from Millipore (Molsheim, France). Tris salt, and all reagent ACS grade were also obtained from Sigma. The kit for the assay of NOS (NOS detectTM) was purchased from Alexis (Alexis Corp., Läufelfingen, Switzerland)

Animals, hormonal regimen and tissue collection

All experiments utilized CL obtained from unmated New Zealand White female rabbits (HY/CR, Charles River Italia, Calco, LC, Italy), age 5 months, weighing 3·5–3·8 kg. The animals were kept at the University of Perugia Central Animal Facility under controlled condition of light (14 h light : 10 h darkness) and temperature (18 °C), and were given water and commercial pellets and allowed to feed *ad libitum*.

The rabbits received an i.m. injection of 20 IU pregnant mare serum gonadotrophin (Folligon, Intervet Italia, Milan, Italy) followed 3 days later by an i.m. injection of 0·8 μg gonadotrophin-releasing hormone (GnRH) analogue (Receptal, Roussel-Hoechst, Munich, Germany) to induce pseudopregnancy. The day of GnRH injection was designated day 0. Rabbits were killed on days 4, 9 and 13 of pseudopregnancy by cervical dislocation. CL were promptly removed from the ovary and, immediately upon collection, were washed with saline and then transferred onto filter paper to dissect away the non-luteal tissue with fine forceps under stereoscopic magnification. For each luteal stage, CL from different rabbits were combined together onto ice-cold medium 199 containing 1% BSA and immediately processed for the *in vitro* study. Additional CL from different rabbits were randomly pooled together on the basis of the day of pseudopregnancy, weighed and stored at −70 °C until assayed for prostaglandin receptors.

In vitro *incubations*

CL from each luteal stage were randomly distributed (one CL/well) into incubation wells containing 1 ml culture medium 199 with Earle's Balanced Salt Solution containing 2·2 mg/ml sodium bicarbonate, 2·3 mg Hepes and 1% BSA, referred to here as M199. For each day of pseudopregnancy, the incubation set of wells was divided into 26 experimental groups. Before treatment, the CL were quartered inside each well using fine forceps. Treatments, performed in four replicate samples, are listed as follows: (1) control (medium alone), (2) PGE_2 (3 μM), (3) PGE_2 plus Gp inhibitor (Gpi, 2 μM), (4) $PGF_{2\alpha}$ (3 μM), (5) $PGF_{2\alpha}$ plus Gpi, and (6) Gpi alone. Additional treatments were (7) Gp activator (Gpa, 0·2 μM), (8) Gpa plus AC inhibitor (ACi 1 μM), (9) Gpa plus PKA inhibitor (PKAi, 2 μM), (10) Gpa plus PLC inhibitor (PLCi, 2 μM),

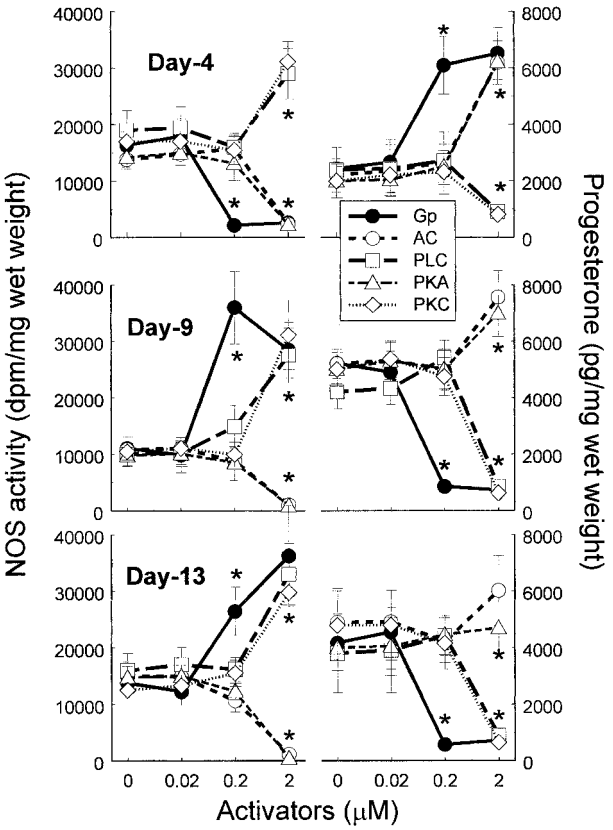

Figure 1 *In vitro* effects of increasing concentrations of activators for Gp (GTPγ-S), AC (forskolin), PLC (imipramine), PKA (8-bromo-cAMP) and PKC (PMA) on total NOS activity (left-hand side) and progesterone release (right-hand side) by rabbit CL collected during early-, mid- and late-luteal phases of pseudopregnancy, on days 4, 9 and 13 respectively, and incubated for 2 h. Values are means ± S.D.S of three determinations. Within each panel, the asterisk indicates significantly different values ($P \leq 0{\cdot}01$ Duncan's multiple range test with respect to other doses of the same compound).

(11) Gpa plus PKC inhibitor (PKCi, 2 μM), (12) Gpa plus NOS inhibitor (NOSi, 1 μM), (13) ACi alone, and (14) PLCi alone. Other treatments were (15) AC activator (ACa, 2 μM), (16) ACa plus PKA inhibitor (2 μM), (17) ACa plus NOSi, (18) PKA activator (PKAa, 2 μM), (19) PKAi, (20) PKAa plus NOSi, (21) PLC activator (PLCa, 2 μM), (22) PLCa plus PKCi, (23) PLCa plus NOSi, (24) PKC activator (PKCa, 2 μM), (25) PKCa plus NOSi, and (26) PKCi alone. The culture plates were incubated at 37 °C in air with 5% CO_2 as reported elsewhere (Gobbetti *et al.* 1999, Boiti *et al.* 2000). CL and media were collected 2 h after the addition of treatments and stored immediately at − 20 °C for later determination of NOS activity and progesterone levels. Minimum doses of activating substances (Fig. 1) and inhibitors used in the present *in vitro* study were defined by preliminary experiments.

NOS activity determination

NOS activity was determined in each CL by monitoring the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline, with a commercial NOS assay kit, according to the experimental protocol as previously described (Boiti *et al.* 2000).

Progesterone assay

Progesterone concentrations in the incubation medium samples were determined following the RIA previously reported (Gobbetti *et al.* 1992). The assay sensitivities were 10 pg/ml, with a working range of the RIA in culture medium of 1–5 pg/ml. Intra-assay and interassay coefficients of variations were 6% and 11% respectively.

Radio-receptor assays

The PGE_2 and $PGF_{2\alpha}$ receptor assays were similar to those reported by Wiepz *et al.* (1992). Briefly, for each day of pseudopregnancy, 50 mg luteal tissue were homogenized in 5 ml ice-cold homogenization buffer (HB: 10 mM Tris–HCl pH 7·0, containing 250 mM sucrose, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0·02% NaN_3) with Polytron PT 1200 for five strokes (5 s × 2500 r.p.m.) interspersed with 20 s cooling periods. The homogenate was centrifuged at 100 ***g*** at 4 °C for 15 min. Pellet was discarded and supernatant was centrifuged at 30 000 ***g*** at 4 °C for 30 min. The resulting pellet was resuspended in 2 ml HB with two strokes of Polytron on ice, and its protein concentration was determined according to the procedure described by Rao *et al.* (1984), using a commercial kit. The concentration and affinity of $PGF_{2\alpha}$ and PGE_2 for specific luteal membrane receptors were assayed by a saturation analysis, using the Multiscreen Assay System device for multiple filtration of unbound radioligands. One hundred micrograms of luteal membrane suspension in 75 μl assay buffer (AB: 10 mM Tris–HCl pH 6·0, containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1·0% BSA and 0·02% NaN_3) were dispensed in triplicate into separate wells of a microtitre glass fibre filter plate. Twenty-five microlitres of five serial dilutions of [^{3}H]$PGF_{2\alpha}$ (2 to 0·125 nM) in AB were then added to each well. The same volume of AB alone or $PGF_{2\alpha}$ (10 μM) was dispensed to total bound (TB) and non-specific binding series of wells (NSB) respectively. The same protocol scheme was repeated to assay PGE_2 receptor, using [^{3}H]PGE_2 and PGE_2 instead of $PGF_{2\alpha}$ at the same molar concentrations. The $PGF_{2\alpha}$ plates were incubated for 2 h at 22 °C, while the PGE_2 plates were kept overnight at 4 °C. At the end of the incubation period each well was washed three times with 300 μl ice-cold saline. The well filters at the bottom of the microplate were carefully blotted on paper, air-dried and cut into separate microvials using the multiple punch assembly of the Multiscreen device. Three millilitres

scintillation fluid were added into each vial and radioactivity was counted on TRICARB 2100 TR liquid scintillation analyser (Packard Camberra).

Specific binding was calculated as the difference between total and non-specific [^{3}H]$PGF_{2\alpha}$ or [^{3}H]PGE_2 binding, normalized to protein content.

Data analysis and statistics

Data for dose–response studies and for effects of treatment on release of progesterone and on basal NOS activity were submitted to ANOVA and Kruskal–Wallis test (Sokal & Rohlf 1981) followed by Duncan's multiple range test for multiple comparison to the control (Duncan 1955). For each day of pseudopregnancy, the individual contrast between treatment groups was made with an unpaired two-tailed Student's *t*-test.

Binding curves were analysed with an iterative, non-linear curve-fitting computer program (PRISM GraphPad Software, San Diego, CA, USA). Data for receptor concentrations (B_{max}) and apparent dissociation constant (K_d) of binding sites are expressed as mean ± S.E.M. Data from [^{3}H]$PGF_{2\alpha}$ and [^{3}H]PGE_2 binding assays were analysed by one-way ANOVA with Dunnett's post test.

Results

The total NOS activity in rabbit CL cultured with medium alone after 2 h *in vitro* incubation was lower ($P \leq 0{\cdot}01$) on day 9 CL (10 886 ± 1170 d.p.m./mg wet tissue) than on day 4 and day 13 CL (15 409 ± 1598 and 14 639 ± 1445 respectively). By contrast, at the end of the incubation period, the corresponding basal progesterone production was higher ($P \leq 0{\cdot}01$) on day 9 CL (4970 ± 245 pg/mg wet tissue) than on CL of day 4 and 13 (2781 ± 284 and 3917 ± 569 respectively).

In all CL preparations, independently of their age, the co-treatment with any of the activators here tested and L-NAME resulted in an inhibition ($P \leq 0{\cdot}01$) of NOS activity and in a concomitant increase ($P \leq 0{\cdot}01$) of progesterone release (Figs 4 and 5).

The dose-dependent effects of increasing concentrations of activators for G proteins, AC and PLC added to the incubation medium on both NOS activity and progesterone secretion in CL of different luteal stages after 2 h *in vitro* incubations are shown in Fig. 1. Independently of the activators here employed, whenever a response was observed the progesterone release was always inversely related ($P \leq 0{\cdot}01$) to the NOS activity.

Effects of prostaglandins on NOS activity and progesterone production by in vitro *cultured CL*

On day 4 CL, PGE_2 addition to the medium, at a concentration of 3 μM, decreased ($P \leq 0{\cdot}01$) NOS activity to 16% of control and caused a twofold increase ($P \leq 0{\cdot}01$) in progesterone production over that of control (Fig. 2). The same treatment, however, was ineffective in both mid- and late-phase CL. By contrast, at the end of the incubation period, $PGF_{2\alpha}$ stimulated a two- to threefold increase ($P \leq 0{\cdot}01$) of NOS baseline activity in rabbit CL tissue preparations of both days 9 and 13 of pseudopregnancy respectively and reduced ($P \leq 0{\cdot}01$) progesterone release to 19–20% of the control (Fig. 2). However, in CL preparation of day 4, the addition of $PGF_{2\alpha}$ did not affect progesterone production and NOS activity, which remained unchanged after the same time interval. The specific and age-dependent selective activation of the prostaglandin signal transduction pathways was blocked by the addition of a Gp inhibitor. In fact, independently of the luteal stages, the co-treatments of CL with Gpi and either PGE_2 or $PGF_{2\alpha}$ failed to induce any response on

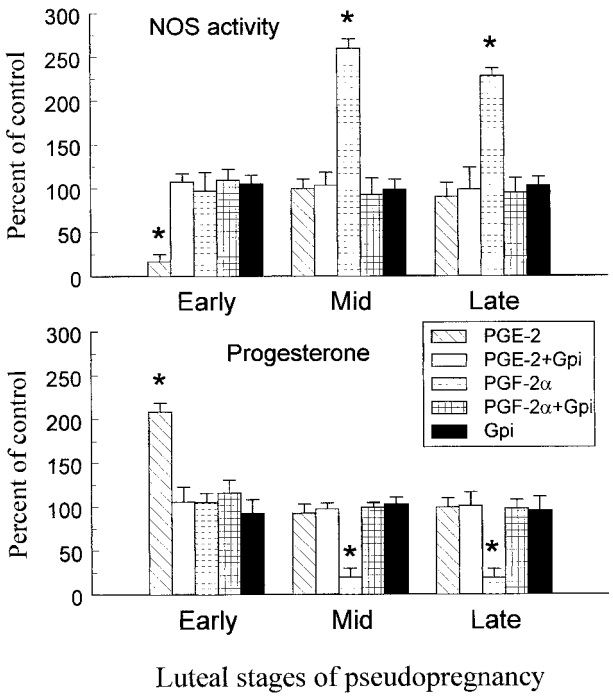

Figure 2 *In vitro* effects of PGE_2 and $PGF_{2\alpha}$ both alone and co-incubated with guanosine 5′-*O*-(2-thiodiphosphate), an inhibitor for G protein (Gpi), and Gpi alone on NOS total activity (upper panel) and progesterone release (lower panel) of rabbit CL collected during early-, mid- and late-luteal stages of pseudopregnancy on days 4, 9 and 13 respectively, after 2-h incubation. Results are the means ± S.D.S of four replicate values and are expressed as a percentage of the control values incubated with medium alone. Means ± S.D.S of NOS activities in the CL incubated with medium alone were 15 409 ± 1598, 10 886 ± 1170 and 14 639 ± 1445 d.p.m./mg wet tissue at days 4, 9 and 13 respectively, while those of progesterone were 2·8 ± 0·3, 4·9 ± 0·3 and 3·9 ± 0·6 ng/mg respectively. Within each panel, an asterisk above the bars of each luteal stage of pseudopregnancy indicates significantly different values ($P \leq 0{\cdot}01$ Duncan's multiple range test).

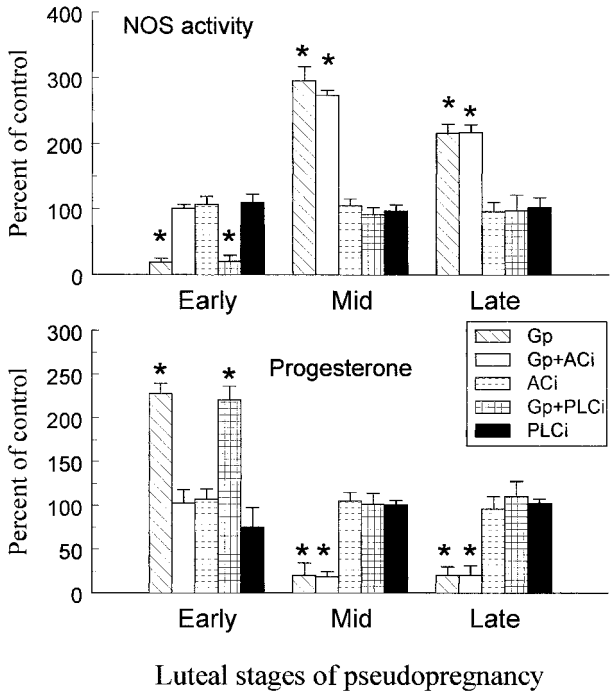

Figure 3 *In vitro* G protein signal transduction effects on NOS total activity (upper panel) and progesterone release (lower panel) of rabbit CL collected during early-, mid- and late-luteal stages of pseudopregnancy on days 4, 9 and 13 respectively, after 2-h incubation. Gp refers to guanosine 5′-*O*-(3-thiotriphosphate), a G protein activator, while ACi and PLCi refer to inhibitors of adenylate cyclase (2-*O*-methyladenosine) and phospholipase C (ET-18-OCH_3) respectively. Results are the means ± S.D.s of four replicate values and are expressed as a percentage of the control values incubated with medium alone. Means ± S.D.s of NOS activities in the CL incubated with medium alone were 15 409 ± 1598, 10 886 ± 1170 and 14 639 ± 1445 d.p.m./mg wet tissue at days 4, 9 and 13 respectively, while those of progesterone were 2·8 ± 0·3, 4·9 ± 0·3 and 3·9 ± 0·6 ng/mg wet tissue respectively. Within each panel, an asterisk above the bars of each luteal stage of pseudopregnancy indicates significantly different values ($P \leq 0{\cdot}01$ Duncan's multiple range test).

either total NOS activity or progesterone production, and had values close to those of the corresponding untreated CL or CL treated with Gp inhibitor alone (Fig. 2).

Effects of in vitro *activation of G protein on NOS activity and progesterone release*

The addition of 0·2 μM GTPγ-S, an activator of the G protein family, induced a fivefold decrease ($P \leq 0{\cdot}01$) of basal NOS activity in day 4 CL, but a two- to threefold increase ($P \leq 0{\cdot}01$) in CL of both day 9 and 13 (Fig. 3). In the same CL preparations, after 2 h *in vitro* incubation, progesterone output exhibited an opposite trend (Fig. 3). In fact, in day 4 CL, steroidogenesis was enhanced ($P \leq 0{\cdot}01$) 228% over that of control as in CL treated with PGE_2. Conversely, in CL of both days 9 and 13, activation of the Gp system caused a marked decrease ($P \leq 0{\cdot}01$) in progesterone production to values similar to those observed following *in vitro* $PGF_{2\alpha}$ treatment.

The co-addition into the culture medium of specific inhibitors for the AC system blocked the Gpa-induced effects on both NOS activity and progesterone release of day 4 CL, while inhibitors of the PLC system did not affect Gpa-mediated effects (Fig. 3). Conversely, in CL of both days 9 and 13, PLC inhibitor counteracted the Gpa-induced effects, while AC inhibitor was ineffective (Fig. 3). Similar luteal stage dependent results were obtained by co-treatments of Gpa with PKA and PKC inhibitors (data not shown). Incubations of CL with AC and PLC inhibitors had no effects (Fig. 3).

Effects of in vitro *activation of AC/PKA system on NOS activity and progesterone release*

The stimulation of the AC/PKA cascade by the addition of either forskolin or 8-bromo-cAMP down-regulated NOS activity in CL of days 4, 9 and 13 (Fig. 4). In day 4 CL, the effects induced by *in vitro* activation of the AC/PKA system overlapped that evoked by PGE_2. By contrast, in mid- and late-luteal phase CL, the inhibition of NOS activity was much higher ($P \leq 0{\cdot}01$) than in day 4 CL and similar to that obtained in CL co-treated with the NOS inhibitor, L-NAME. On day 4 CL, the effects of both AC and PKA activators mimicked PGE_2 treatment by increasing basal progesterone secretion 223% over that of control, while on day 9 CL the luteotrophic effect was less effective as progesterone production rose ($P \leq 0{\cdot}01$) only 50% over that of controls (Fig. 4). Conversely, in day 13 CL progesterone release remained unaffected by treatments with both AC and PKA activators. In CL of all ages, the co-treatment with PKA inhibitor abolished the luteotrophic effect induced by the AC activator by restoring NOS activity and progesterone release to their respective basal control values (Fig. 4). Treatment with PKA inhibitor alone had no effect on both NOS activity and progesterone release (Fig. 4).

Effects of in vitro *activation of PLC/PKC system on NOS activity and progesterone release*

The *in vitro* treatments with either the PLC or the PKC system activator greatly decreased ($P \leq 0{\cdot}01$) progesterone production by CL preparations independently of their age (Fig. 5). The effects on the NOS activity induced by these treatments were opposed and caused a two- to threefold increase ($P \leq 0{\cdot}01$) of basal NOS activity over that of controls (Fig. 5). The co-addition of the PKC inhibitor to CL treated with PLC activator abolished the luteolytic effect and the up-regulation of NOS activity. Similar results were obtained by treatment with PKC inhibitor

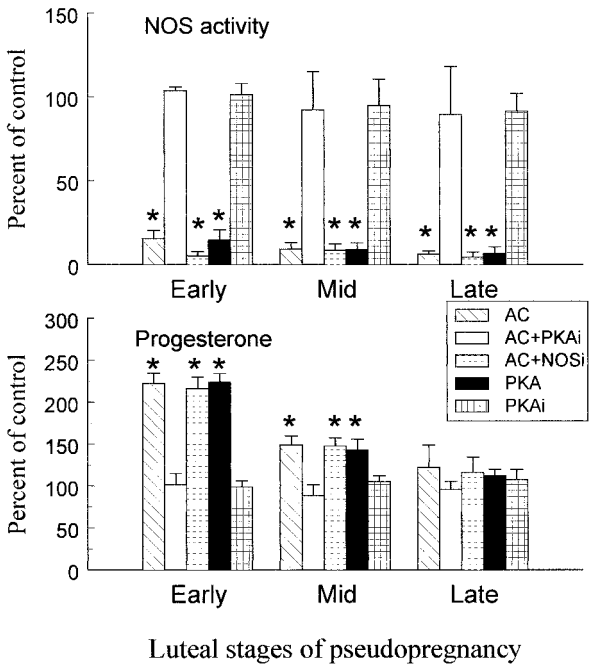

Figure 4 *In vitro* activation of the AC/PKA system on NOS total activity (upper panel) and progesterone release (lower panel) of rabbit CL collected during early-, mid- and late-luteal stages of pseudopregnancy on days 4, 9 and 13 respectively after 2-h incubation. AC and PKA refer to activators of adenylate cyclase (forskolin) and protein kinase A (8-bromo-cAMP), while PKAi and NOSi refer to inhibitors of PKA (compound H89) and NOS (L-NAME) respectively. Results are the means ± S.D.s of four replicate values and are expressed as a percentage of the control values incubated with medium alone. Means ± S.D.s of NOS activities in the CL incubated with medium alone were 15 409 ± 1598, 10 886 ± 1170 and 14 639 ± 1445 d.p.m./mg wet tissue at days 4, 9 and 13 respectively, while those of progesterone were 2·8 ± 0·3, 4·9 ± 0·3 and 3·9 ± 0·6 ng/mg wet tissue respectively. Within each panel, an asterisk above the bars of each luteal stage of pseudopregnancy indicates significantly different values ($P \leq 0{\cdot}01$ Duncan's multiple range test).

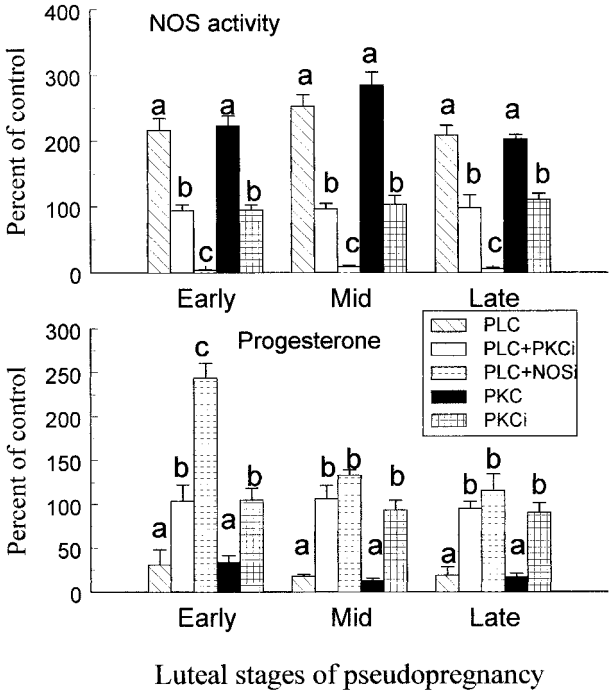

Figure 5 *In vitro* activation of the PLC/PKC system on NOS total activity (upper panel) and progesterone release (lower panel) of rabbit CL collected during early-, mid- and late-luteal stages of pseudopregnancy on days 4, 9 and 13 respectively after 2-h incubation. PLC and PKC refer to activators of phospholipase C (imipramine) and protein kinase C (PMA), while PKCi and NOSi refer to inhibitors of PKC (staurosporine) and NOS (L-NAME) respectively. Results are the means ± S.D.s of four replicate values and are expressed as a percentage of the control values incubated with medium alone. Means ± S.D.s of NOS activities in the CL incubated with medium alone were 15 409 ± 1598, 10 886 ± 1170 and 14 639 ± 1445 d.p.m./mg wet tissue at days 4, 9 and 13 respectively, while those of progesterone were 2·8 ± 0·3, 4·9 ± 0·3 and 3·9 ± 0·6 ng/mg wet tissue respectively. Within each panel, different letters above the bars of each luteal stage of pseudopregnancy indicate significantly different values ($P \leq 0{\cdot}01$ Duncan's multiple range test).

alone. In fact, both progesterone release and total NOS activity remained unchanged, having values close to those found in control CL of the same ages (Fig. 5).

Binding affinities and concentrations of $PGF_{2\alpha}$ and PGE_2 receptors

Saturation curves and Scatchard plot transformations derived by binding isotherms for each prostaglandin in the different luteal stages are shown in Fig. 6. Independently of the luteal stages, the concentrations of receptor sites for $PGF_{2\alpha}$ were greater than the concentrations for PGE_2 receptors, but increased markedly over those for PGE_2 from twofold on day 4 CL to 15-fold on CL of both mid- and late-luteal stages (Fig. 7). The concentration of receptors for $PGF_{2\alpha}$ increased ($P \leq 0{\cdot}01$) four- to fivefold from early to mid- and late-luteal phase, being highest on day 13 CL. By contrast, on day 4 CL the concentration of receptors for PGE_2 was highest ($P \leq 0{\cdot}01$) compared with lower values found in older CL. In CL of mid-luteal phase, the dissociation constants (K_d) for the receptors for PGE_2 and $PGF_{2\alpha}$ were 1·6 ± 0·5 and 1·9 ± 06 nM respectively. Similar values were obtained also in CL of early and late-luteal phases. For each prostaglandin, slope factors for the competition curves were not statistically different from 1, suggesting a ligand receptor interaction that could be explained by a one-site binding model.

Discussion

Although the involvement of receptors for both $PGF_{2\alpha}$ and PGE_2 has been postulated in CL of pseudopregnant

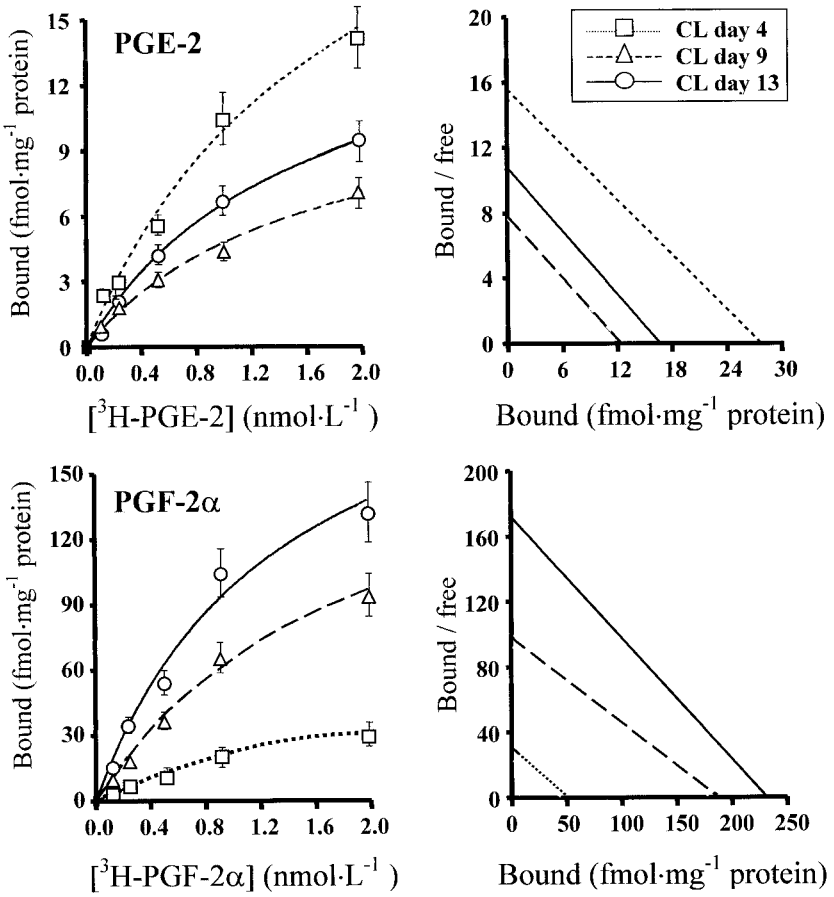

Figure 6 Representative saturation curves (left side) and Scatchard plots (right side) from one of three independent experiments of [^{3}H]PGE_2 (upper panels) and [^{3}H]$PGF_{2\alpha}$ (lower panels) binding to luteal membrane fraction homogenates prepared from pooled rabbit CL collected at the early-, mid- and late-luteal phase on days 4, 9 and 13 of pseudopregnancy. Values are the means ± S.E.M.S.

rabbits on the basis of their luteolytic and luteotrophic actions (Boiti *et al.* 2000), this work is the first direct evidence for their existence in this species. Independently of CL age, luteal cells have now been clearly shown to display luteal membrane binding sites for these PGs. Under the assay conditions used for this study, the binding characteristics suggest a one-site model for each receptor. However, while the capacity of $PGF_{2\alpha}$ receptors increased markedly over time with the ageing of CL, by contrast the number of binding sites of the PGE_2 receptors decreased progressively from early- to late-luteal phase.

Several studies have identified receptors for both PGE_2 and $PGF_{2\alpha}$ and their distribution in luteal cells of different species including laboratory rodents and farm animals. Wright *et al.* (1980) found that affinity and capacity of $PGF_{2\alpha}$ receptors in CL of rats do not change across pseudopregnancy. High-affinity $PGF_{2\alpha}$ binding sites have been found in bovine CL already in the early luteal phase (Sakamoto *et al.* 1995, Wiltbank *et al.* 1995) when CL are usually resistant to single treatment with exogenous $PGF_{2\alpha}$. Moreover, Sakamoto *et al.* (1994, 1995) showed that the expression of mRNA for luteal membrane $PGF_{2\alpha}$ receptors and the number and affinity of their binding sites increased only slightly during the oestrous cycle of the bovine. These findings differ from those previously reported by Rao (1974), who demonstrated a marked increase in the affinity of the $PGF_{2\alpha}$ receptor of bovine CL, which parallels the increase in sensitivity to $PGF_{2\alpha}$-induced luteolysis during the bovine oestrous cycle. In pigs, PGE_2 receptor concentration appeared to be related to CL functional status during the oestrous cycle (Feng & Almond 1996). Therefore, considerable differences among species exist in the ways receptors for PGs change during the time-course of the CL life span, a feature that adds complexity to the underlying regulatory mechanisms which probably involve also intraluteal factors (Skarzynski & Okuda 1999).

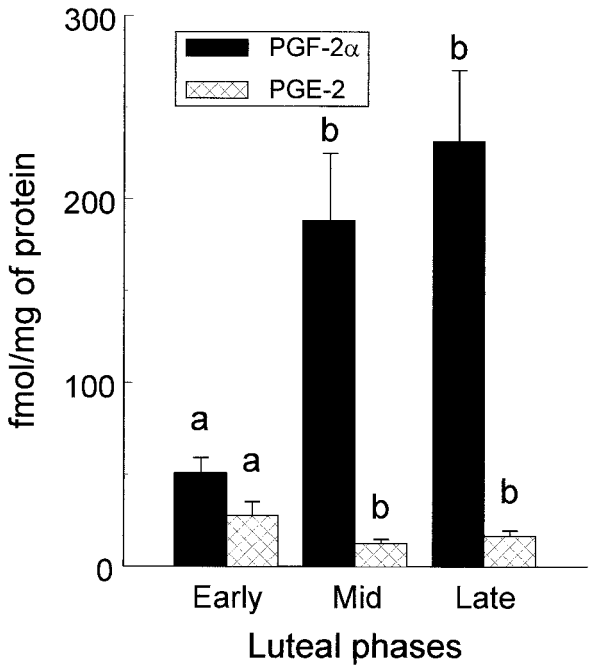

Figure 7 Concentrations (means ± S.E.M.) of $PGF_{2\alpha}$ and PGE_2 receptors in CL of rabbits collected at the early-, mid- and late-luteal phase on days 4, 9 and 13 of pseudopregnancy. Within each series, different letters above the bars indicate significantly different values ($P \leq 0{\cdot}01$ Dunnett's post test).

Several reports suggest a role of G proteins in the signalling pathways involved in the transduction of prostaglandin actions. The effects of $PGF_{2\alpha}$-receptor activation appear to be transduced by the Gp family and those of PGE_2 by interaction with a stimulatory Gp (Olofsson & Leung 1996). This role has been indirectly confirmed here by the lack of specific prostaglandin-induced responses following the co-application of Gp inhibitors and either PGE_2 or $PGF_{2\alpha}$ to CL *in vitro* and by the mimicking effects obtained by the addition of Gp activators. The Gp-dependent AC/PKA pathway, regulating NOS enzymatic activity and steroidogenesis, was triggered only on day 4 CL. In older CL, the Gp-activated PLC/PKC pathway prevailed. In CL of both days 9 and 13, activation of the G protein system evoked a marked luteolytic effect similar to that observed following treatment with $PGF_{2\alpha}$. This prostaglandin has been shown to mediate functional luteolysis and luteal regression in the mammalian ovary, with the relevant exception of man and other primates (Niswender *et al.* 2000). In ovaries from both human and other primate species, however, $PGF_{2\alpha}$ does indeed activate the phospholipase C signalling pathway (Davis *et al.* 1989). The luteolytic action of $PGF_{2\alpha}$ in rabbit CL depends upon the availability of functional receptors on target luteal cells and, following receptor binding, appears to be mediated by several mechanisms integrating a number of second messengers such as PLC and PKC phosphorylation in signal transduction. Prostaglandin $F_{2\alpha}$-receptor occupations in both day 9 and day 13 CL cultured *in vitro* rapidly stimulated total NOS activity and inhibited progesterone, thus confirming our previous results (Gobbetti *et al.* 1999, Boiti *et al.* 2000). In our model, independently of the luteal stages, whenever total NOS-like activity was significantly up-regulated, a functional luteolytic response was observed as evidenced by a marked reduction of progesterone production. The signalling pathway leading to luteolysis was interrupted by incubation with L-NAME. These findings strongly support the hypothesis for a functional role of NO in the $PGF_{2\alpha}$-mediated luteolytic process in rabbit CL. A key role in the regulation of NOS activity should be attributed to Ca^{2+} mobilization, which increases its free intracellular levels following $PGF_{2\alpha}$-receptor occupation. In fact, it is well established that constitutive endothelial NOS requires Ca^{2+} for the reversible binding of calmodulin to the enzyme to become active and generate NO (Bredt & Snyder 1990). $PGF_{2\alpha}$ rapidly induces an increase in phosphoinositide turnover by PLC and, consequently, a rise in intracellular diacylglycerol (DAG) and inositol triphosphate (InP_3) levels; DAG activates PKC while InP_3 frees intracellular Ca^{2+} (Wiltbank *et al.* 1991). Conversely, the activation of the receptor for PGE_2, EP_2 is associated with elevation of intracellular levels of cAMP (Olofsson & Leung 1986).

Studies of prostaglandin actions *in vitro* have often yielded contradictory results on the production of progesterone. In contrast to our present findings, McLean *et al.* (1987) reported that neither $PGF_{2\alpha}$ nor PGE_2 altered progesterone secretion by dispersed luteal cells obtained from rabbits at day 10 of pseudopregnancy. Dharmarajan *et al.* (1989) found that $PGF_{2\alpha}$ did not affect progesterone production in the *in vitro* perfused rabbit ovary *in vitro* at day 11 of pseudopregnancy. These discrepancies are mainly related to the difference in the *in vitro* technique used. It remains to be established, however, if the increased progesterone release is due to an actual synthesis rather than to a leakage from dying cells as suggested by Dharmarajan *et al.* (1994, 1999) who reported that rabbit CL undergo spontaneous apoptosis within a few hours when cultured in serum-free medium.

The luteolytic action of $PGF_{2\alpha}$ was clearly dependent on the age of the CL. Four-day-old CL remained unaffected by the same luteolytic dose of $PGF_{2\alpha}$ which caused a striking reduction in progesterone production by mature CL. On the basis of the binding studies here reported, the age-dependent unresponsiveness of young CL may be the combined results of fewer functional receptors available for $PGF_{2\alpha}$ associated with an increase in the number of receptors for PGE_2 conveying luteotrophic or antiluteolytic signals. In fact, it has been shown that $PGF_{2\alpha}$ can bind to receptors for PGE_2 (Christenson *et al.* 1994, Coleman 1996). Although the low receptor density for $PGF_{2\alpha}$ may well explain the resistance to this prostaglandin in the early luteal stage, an impairment of Gp

coupled to $PGF_{2\alpha}$ receptor could not be ruled out. In fact, the addition into the culture medium of activators for either PLC and PKC enzymes caused a marked luteolytic effect not only on day 9 and day 13 CL, similar to that obtained by $PGF_{2\alpha}$ treatment, but also in day 4 CL, when the same prostaglandin was completely ineffective. The increasing $PGF_{2\alpha}$ receptor density occurring in the mid- and late-luteal stages of pseudopregnancy is consistent with the luteolytic effects observed *in vitro*, which involve interaction of the CL and $PGF_{2\alpha}$ itself. Rabbits, however, are completely refractory to $PGF_{2\alpha}$ injection until day 12 of pseudopregnancy (Marcinkiewicz *et al.* 1992), even if they exhibit earlier responsiveness to the $PGF_{2\alpha}$ analogue, alfaprostol (Boiti *et al.* 1998). The lack of response to $PGF_{2\alpha}$ *in vivo* before day 12 of pseudopregnancy is therefore unlikely to be caused by a reduced expression of binding sites for $PGF_{2\alpha}$, and suggests the involvement of other factors.

In several species, PGE_2 has been identified as a luteotrophic factor and it is supposed to counteract $PGF_{2\alpha}$-induced luteolysis (Henderson *et al.* 1977, Silvia *et al.* 1984). PGE_2 has been involved in the maintenance of the CL during early pregnancy when progesterone secretion from the CL is essential for the establishment and maintenance of pregnancy (Christenson *et al.* 1994). According to Grimes *et al.* (1993), PGE_2 enhances progesterone synthesis via cAMP stimulation, as well as the expression of the genes for both steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and mitochondrial cytochrome $P450_{scc}$ (Li *et al.* 1993). Our *in vitro* study demonstrated direct PGE_2-receptor-mediated effects in CL of pseudopregnant rabbits only in the early luteal phase. In fact, PGE_2-stimulated progesterone production was abolished by co-incubation with Gp inhibitor, while similar results were obtained when Gp activator was used. On day 4 CL, the action on luteal cell steroidogenesis following binding is exerted at multiple sites and probably involves AC and PKA up-regulation. In fact, cAMP-derivative and PKA-activator likewise stimulated progesterone releases by CL of the same age. That the effect is at least partially exerted through cAMP-mediated mechanisms is further supported by the findings that PGE_2-induced progesterone release was abolished by co-treatments with inhibitors of the AC/PKA systems. As previously stated, the stimulatory action of PGE_2 was found to be present only on day 4 CL, while, thereafter in mature CL, a positive response was not observed. Interestingly, a similar differential responsiveness to PGE_2 has also been reported in monkey; incubation with this prostaglandin increased progesterone production by luteal cells obtained from CL in the early phase of the menstrual cycle on days 3–5, but was ineffective in subsequent stages (Brannian & Stouffer 1991). However, the absence of any PGE_2-induced luteotrophic effects in rabbit CL at days 9 and 13 seems not to be associated with a complete lack of luteal membrane PGE_2 receptors. The unresponsiveness of both day 9 and day 13 CL to PGE_2 could also be ascribed to prior *in vivo* desensitization to PGE_2 receptor or to receptor uncoupling from AC. Taken together, these results suggest that PGE_2 may not have a relevant role in CL of either mid- or late-luteal stages of pseudopregnancy. However, even if PGE_2 enhances progesterone production only in the early luteal phase, AC/cAMP and PKA second messenger systems, working in a luteotrophic or luteoprotective fashion, are present in CL of both mid- and late-luteal phases. Notably, maximal stimulation of progesterone production occurred on day 9 CL in the presence of AC and PKA activators. Even if 17β-oestradiol has been identified as the principal luteotrophic hormone, rabbit CL exhibit LH receptors, LH-responsive AC and a cAMP-dependent protein kinase enzyme system which is specifically activated in a time- and a dose-dependent manner (Hunzicker-Dunn & Birnbaumer 1976, Hoyer *et al.* 1986).

In the present study we demonstrated that the receptor capacity of both $PGF_{2\alpha}$ and PGE_2 changes in CL of pseudopregnant rabbits during the luteal phases. However, the factors that may influence the increased expression of $PGF_{2\alpha}$ receptor during pseudopregnancy while reducing that of PGE_2, remain to be explored. Differential binding capacities of $PGF_{2\alpha}$ and PGE_2 receptors between early- and both mid- and late-luteal stages may reflect functional differences not yet understood and in this respect it would be interesting to compare their characteristics in relation to physiological conditions of pregnancy and pseudopregnancy. The mechanisms by which the CL of rabbit are refractory to the luteolytic action of $PGF_{2\alpha}$ during the early period of pseudopregnancy remain to be disclosed and better characterized, but are probably mediated by factors other than a complete lack of luteal receptor–ligand interactions. The combined results of this study provide further evidence that the contrasting prostaglandin-induced luteolytic and luteotrophic effects observed in rabbit CL cultured *in vitro* are mediated by different receptors for $PGF_{2\alpha}$ and PGE_2 coupled to Gp families. Moreover, this study confirms that PLC/PKC and AC/PKA systems play an important role in the transduction of signals regulating prostaglandin-induced luteolytic and luteotrophic effects respectively.

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