Functional lysophosphatidic acid receptor in bovine luteal cells

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Abstract The aim of this study was to determine whether luteal cells possess functional receptors for lysophosphatidic acid (LPA). We present evidence that [^{3}H]LPA binds to a 38–40 kDa protein in a membrane fraction prepared from luteal cells and that this phospholipid is able to induce tyrosine phosphorylation of several proteins (65–125 kDa). Furthermore, LPA upregulates forskolin- and LH/GTP-stimulated adenylyl cyclase activity by changing its Vmax. Although a pertussis toxin-sensitive G-protein has been reported to transmit the inhibitory signals between the LPA receptor and adenylyl cyclase, the observed upregulation of the enzyme activity in luteal cells is not abolished after pre-treating the cells with the toxin, suggesting that a different mechanism is operative in these cells. According to the pharmacological regulatory pattern it is suggested that the modulated adenylyl cyclase isoform is the enzyme subtype V expressed in luteal cells.

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Key words: Lysophosphatidic acid; Lysophosphatidic acid receptor; Tyrosine phosphorylation; Cyclic AMP; Adenylyl cyclase type V; Ovary

1. Introduction

Lysophosphatidic acid (LPA) is a biologically active phospholipid produced upon activation of secretory phospholipase A2 and hydrolysis of pre-existing phospholipids during platelet activation [1]. Though only freshly prepared platelet-rich plasma contains high amounts of LPA (2–20 μM), its production and release is not restricted just to platelets [1–3]. LPA may also be formed and released by several types of injured cells [3] and it is present both in ascites from ovarian cancer cells [4] and in human follicular fluid [1].

This phospholipid is responsible for a variety of reported serum effects and has the potential to modulate cellular signalling in various cell types determining their shape and differentiation status [2,3]. Since LPA does not penetrate cells, it could either be metabolised in the cell membrane or bring about its effects upon binding to a receptor. Its biological activity has not yet been shown to be attributed to one of its metabolites. However a G-protein-coupled seven transmembrane receptor, identified by photoaffinity labelling and by molecular cloning, has been proposed as a candidate cell surface receptor for LPA [2,3,5–7].

Upon binding to its receptor, LPA has been shown to activate several Ser/Thr kinases [8] and to cause rapid tyrosine phosphorylation of 110–130, 95 and 65–75 kDa proteins [8–10]. Functionally LPA is known to induce at least four G-protein-mediated signal transduction cascades such as the stimulation of phospholipases C and D, inhibition of adenylyl cyclase and activation of ras [2,3]. Recent studies show that exposure of cells to LPA results in a modulation of their abilities to respond to hormones that interact with G-protein-linked receptors [11,12]. In the ovary, both steroid production and the state of cellular differentiation are regulated primarily by the gonadotropin receptor-coupled adenylyl cyclase. Modulation of this enzyme may, therefore, have a major consequence for the regulation of various differentiated cell functions in this system. We have reported previously [13] that treatment of bovine luteal cells with bacterial phospholipase D can lead to an enhancement of forskolin- and LH-stimulated adenylyl cyclase activity. Since bacterial phospholipase D has been shown to trigger its effects through activation of the LPA receptor [3], it was of interest to determine whether functional binding sites for LPA are present in luteal cells. Furthermore, possible biological responses to LPA in this cell system have also been evaluated.

2. Materials and methods

2.1. Materials

The sources of chemicals were as follows: 1-oleoyl-LPA from Sigma (Deisenhofen, Germany), [^{3}H]oleoyl-LPA from NEN (Köln, Germany), PTX from List Biological Laboratories Inc (Campbell, CA), TLC plates from Whatman (Maidstone, England), TLC solvents from Merck (Darmstadt, Germany), cyclic AMP assay reagents were provided by IBL Immunochemicals (Hamburg, Germany), peroxidase-based Western blot detection systems from Pierce (provided from KMF Sankt Augustin, Germany) and alkaline phosphatase detection system Tropix from Serva (Heidelberg, Germany). Bovine LH was a gift from NIADDK (Bethesda, MD). Anti-phosphotyrosine antibody was from Upstate Biotechnology (Biomol, Hamburg, Germany), anti-adenylyl cyclase antibodies were either a gift from Dr. Droste (Institute for Physiological Chemistry II, Heinrich-Heine University, Düsseldorf, Germany) or were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Secondary antibodies were from Dianova (Hamburg, Germany). All other reagents were obtained commercially and were of the highest purity grade.

2.2. Cell culture and incubation

The method for the isolation and purification of bovine luteal cells has been published elsewhere [13,14]. The cells were maintained in DMEM/HAM's F-12 Medium (incl. 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated foetal calf serum). On the second day, the medium was replaced by fresh medium supplemented with 5 μg/ml bovine serum albumin, 5 μg/ml transferrin, 5 μg/ml insulin, 5 ng/ml sodium selenite and the cells were cultured further for 72 h before treatment. On day 5, culture medium was removed from the confluent cell monolayers and the cells were incubated with or without LPA in serum-free medium containing 0.1% BSA. Unless otherwise stated, the cells were treated with 50 μM LPA for 15 min. The aspirated conditioned medium was used for further analysis, and the cell pellet was stored frozen for further treatments (i.e. the membrane preparation).

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Abbreviations: LPA, lysophosphatidic acid (1-alkyl-glycero-3-phosphate); PTX, pertussis toxin; LH, luteotropic hormone

This paper is dedicated to Prof. F.A. Leidenberger, the founder of the Institute for Hormone and Fertility Research, on the occasion of his 60th birthday.

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2.3. Treatment of luteal membranes with LPA in vitro

Preparation of luteal membrane fractions was performed as described [13]. Their protein content was determined according to Bradford [15]. The 100 000 × *g* membrane fractions were incubated in 100 µl with PIPES buffer, pH 7.0 in the presence of 10 mM $MgCl_2$, 50 µM $CaCl_2$, 50 µM EGTA, 100 µM ATP and various concentrations of oleic LPA. The samples were immediately centrifuged at 40 000 × *g* at 4°C for 60 min. The resulting pellet was used in adenylyl cyclase enzyme assay.

2.4. Adenylyl cyclase assay

The enzyme assay was carried out at 30°C for 15 min in a previously described [13] standard assay incubation mixture (100 µl) including an ATP regenerating system, 1 mM ATP (or various ATP concentrations for kinetic analysis), 5 mM $MgCl_2$ and 10 µg membrane protein equivalent as indicated. After ethanol extraction, the amounts of cyclic AMP formed were measured either by radioimmunoassay as described previously [13,14] or by a specific cAMP-ELISA (IHF GmbH, Hamburg). The same anti-cAMP antibody [13,14] was used for either radioimmunoassay or ELISA. Enzyme kinetics were analysed according to the Lineweaver-Burk method.

2.5. Analysis of [^{3}H]LPA binding

Membrane fractions were incubated with [^{3}H]LPA in the presence and absence of cold LPA and 0.5 mM $CuSO_4$ with 20 mM Tris, pH 7.5. The mixture (100 µl) was incubated for 60 min at 30°C after which 0.5 µM disuccinimidyl suberimate (DSS) was added to cross-link LPA to its binding protein, and the membranes were incubated 20 min at 4°C. Before heating in Laemmli sample buffer solubilising buffer was added (20 mM Tris pH 7.5, 1% SDS, 1 mM) and the samples were subjected to SDS-PAGE analysis. LPA cross-linked to its receptor protein was visualised with a help of a Berthold linear analyser system. The peak fractions were cut out of the gel, and the amounts of the bound radioactivity were counted in a beta counter.

2.6. Assessment of [^{3}H]LPA metabolism

The membrane fractions were incubated with [^{3}H]LPA in assay buffer (see binding) with and without $CuSO_4$ for 30 min at 30°C. The radioactive lipids were extracted from the membranes by the method of Bligh and Dyer [16] as described elsewhere [13]. The organic phases were dried, redissolved in 50 µl of $CHCl_3/CH_3OH$ (9:1) and applied to silica gel G 60 linear high performance thin layer plates (HPTLC). The plates were developed using two different solvent systems: first system consisting of chloroform/methanol/glacial acetic acid/water 75:48:12.5:3.5 [16] and the second one consisting of chloroform/methanol/acetic acid 65:15:2 [17,18] permitting the separation of LPA from phosphatidic acid, phospholipids and neutral lipids. Lipids were located by staining the standards with iodine vapour and autoradiography. The silica gel areas of interest were scraped and the amounts of the radioactivity extracted from these areas were measured in a scintillation counter.

2.7. Analysis of phosphotyrosine phosphorylation

The membrane fractions separated by SDS-PAGE and transferred onto PVDF membrane were blocked overnight followed by the incubation with polyclonal anti-phosphotyrosine antibody overnight. The membrane was washed and incubated with peroxidase conjugated anti-rabbit secondary antibody for 60 min. The immunocomplex was detected using a peroxidase-based chemiluminescence detection system. No staining could be observed when rabbit non-immune serum was applied as control.

2.8. Expression of adenylyl cyclase V protein in bovine luteal cells

Membranes were separated by SDS-PAGE, blocked and incubated with either polyclonal rabbit antibody recognising the peptide sequence corresponding to the 5′ end of the coding region of the adenylyl cyclase type V [19] or a polyclonal antibody mapping the amino acids 1019–1051 of the carboxy terminus of rat adenylyl cyclase V according to the protocol described above. The immunocomplex was detected using a peroxidase-based chemiluminescence detection system. In some experiments alkaline-phosphatase conjugated monoclonal antibody recognising all adenylyl cyclase isoforms (BBC2.AP) was used. The immunoreactive bands were visualised with Tropix-CDP-Star solution. To avoid tissue-dependent non-specific reaction the membranes from rat heart were used as a positive control.

2.9. Data presentation and analysis

The statistical analyses (ANOVA and *t*-test) were carried out using GraphPAD Intuitive Software for Science (San Diego, CA). If not otherwise stated, the results are presented as mean ± SE (triplicate determinations) in a single representative experiment. Error bars have been omitted where these were smaller than the symbols. Each experiment was performed at least three times.

3. Results

3.1. [^{3}H]LPA binds specifically to luteal membrane fraction

Based on radioligand receptor cross-linking experiment, we show here that [^{3}H]LPA is able to bind to a 38–40 kDa protein in luteal cell membranes (Fig. 1). Addition of unlabelled LPA could displace the binding of the radioligand in a concentration-dependent manner (Fig. 1).

In order to determine to what extent the radioligand could be enzymatically degraded by lysophospholipases or acyltransferases in bovine luteal cells, we incubated the membrane fraction with [^{3}H]LPA for 60 min after which lipids were extracted and separated by HPTLC (Fig. 2). The amounts of radioactivity associated with phosphatidic acid (PA) and lysophosphatidic acid were determined. After 60 min of incubation, 26–28% of the radioactivity was associated with LPA, < 9% was converted to PA, 45–50% to monoacylglycerol (MAG) and 10–14% to free fatty acid.

3.2. LPA stimulates tyrosine phosphorylation of luteal membrane proteins

Addition of LPA has been reported to cause tyrosine phosphorylation of multiple substrates in Swiss 3T3 cells and in rat fibroblasts [9,11,20,29]. To elucidate the functionality of LPA binding in luteal cells, it was of interest to see whether LPA is able to induce protein tyrosine phosphorylation in these cells. As shown in Fig. 3, addition of 1 µM LPA is sufficient to stimulate phosphorylation of several membrane proteins. The most dramatic increase of phosphorylation on tyrosine residues is observed in the kDa range from 65–125.

3.3. LPA sensitises both LH- and forskolin-stimulated adenylyl cyclase activity in vitro

To test whether LPA treatment could directly affect luteal

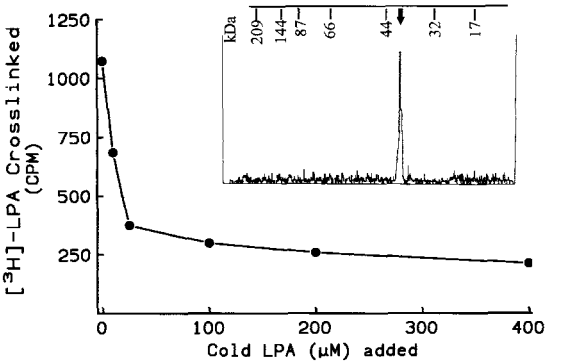


Fig. 1. [^{3}H]LPA binding to a 38 kDa membrane protein in bovine luteal cells. Membrane fractions were incubated with [^{3}H]LPA and indicated amounts of cold LPA for 60 min at 30°C, after which the cross-linking of the radioligand receptor complex with disuccinimidyl suberimate and electrophoretic separation of membrane proteins were performed. The gel was scanned (A) and the amounts of the radioactivity in peak fractions were measured in a beta-counter (B).

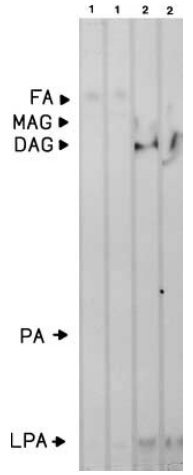


Fig. 2. Assessment of [^{3}H]LPA metabolism. Membrane fractions were incubated with [^{3}H]LPA (0.5 nM) for 60 min (see Fig. 1). The radioactive membrane lipids were extracted and separated on HPTLC plates (see Section 2). Lane 1 (run in duplicate) shows the methanol/water fraction and lane 2 (duplicate) the chloroform fraction.

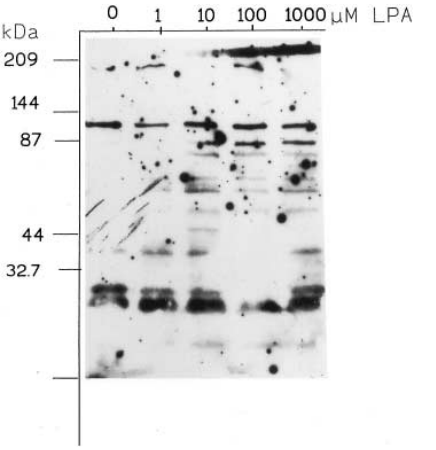


Fig. 3. LPA-induced protein tyrosine phosphorylation in luteal cells. Membrane fractions were prepared from luteal cells treated with or without different LPA concentrations (1–1000 μM). The samples were run on SDS-PAGE, blotted onto PVDF membrane and hybridised with specific anti-phosphotyrosine antibody.

adenylyl cyclase activity, membrane fractions from LPA-treated and untreated control cells were prepared and the enzyme activity was measured. As shown in Fig. 4, pre-treatment of luteal cells with LPA resulted in a marked enhancement of LH/GTP-stimulated adenylyl cyclase activity. This enhancing effect of LPA was dose-dependent; half maximal stimulation occurred with 800 nM LPA and a maximum effect was observed with 150–200 μM, diminishing thereafter with higher concentration of LPA (Fig. 4A). Kinetic analysis revealed that the observed sensitisation of the enzyme activity resulted from a change in Vmax (7.28 versus 4.48 pmol/mg protein/min for LPA-treated versus untreated cells) with no effect on the Km of the enzyme (0.26 and 0.28 for treated and untreated cells respectively).

Instead of intact cells, if membrane fractions were first prepared and then treated with LPA an even more pronounced effect on both LH- and forskolin-stimulated adenylyl cyclase activity could be observed (Fig. 4B). Interestingly in this case, the stimulatory effect of LPA was dose-dependent and, unlike the cells, no inhibitory effect was observed even with as high a concentration of LPA as 400 μM (solubility limited the use of higher LPA concentrations).

3.4. Pertussis toxin does not affect LPA-induced sensitisation of adenylyl cyclase

LPA has been reported to cause both inhibition [21] and sensitisation [22] of adenylyl cyclase activity, and either of these effects could be completely abolished following PTX pre-treatment. Therefore, we have examined if this is the case for luteal cells as well. As shown in Fig. 5, treatment of the cells with LPA alone increased the rate of LH/GTP-stimulated adenylyl cyclase reaction. Prior exposure of the cells to pertussis toxin caused a small augmentation of the enzyme activity. The sensitising effect of LPA in luteal cells was not blocked by pertussis toxin. On the contrary, an augmentation was observed. This might be due to a different type of adenylyl cyclase in luteal cells compared to those used in other reported studies.

3.5. Bovine luteal cells express type V adenylyl cyclase

Since there is absolutely no information in the literature as to which adenylyl cyclase isoform is present in the ovary, we demonstrate in the following set of experiments that adenylyl cyclase type V is present in luteal membranes. Based on Western blotting procedure, we used at the outset a monoclonal antibody recognising several cyclase isoforms (Fig. 6, lanes 1 and 2), then a polyclonal antiserum [19] recognising the peptide sequence corresponding to 5′ end of the coding region of the adenylyl cyclase type V (lanes 3 and 4) and finally polyclonal antibody raised against the carboxy terminus of rat adenylyl cyclase type V corresponding to amino acids 1019–1051 (lanes 5 and 6). As a control (lane 7), membranes from rat heart, known to express type V adenylyl cyclase [19,24] were used. The results in Fig. 6 show that luteal cells appear to express a 150 kDa protein recognised by a non-selective

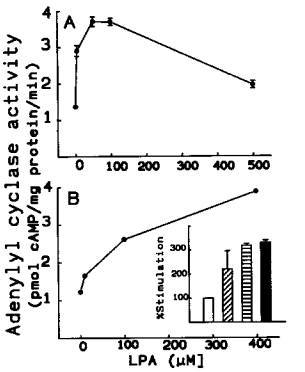


Fig. 4. Effects of pre-incubation of luteal cells (A) or membranes (B) with LPA on LH receptor-coupled adenylyl cyclase activity. A: Luteal cells were treated with various LPA concentrations for 15 min and the membrane fractions were used in adenylyl cyclase assay performed in the presence of 100 ng/ml bovine LH and 10 μM GTP. B: Membrane fractions were incubated as described in Section 2 with either various LPA concentrations or with 100 μM LPA (inset). The samples were centrifuged at 40 000×g and the resulting pellet was used in adenylyl cyclase assay performed in the presence of either buffer (no fill in), GTP (diagonal), LH/GTP (● and horizontal) or forskolin (solid).

anti-adenylyl cyclase antibody as well as by two different anti-adenylyl cyclase type V-specific antibodies indicating that luteal cells express this adenylyl cyclase isoform.

4. Discussion

The data presented here demonstrate that [^{3}H]LPA binds to a 38–40 kD protein in luteal cell membranes and that this binding results in tyrosine phosphorylation of several membrane proteins in the molecular weight range of 65–125 kDa. The lipid analysis of the cell fractions labelled with [^{3}H]LPA shows clearly that it is metabolised to a variety of products. Of these, conversion to [^{3}H]PA can be considered of only minor importance, suggesting that the observed LPA effects can not be explained by its conversion to phosphatidic acid. We observed further that pre-treatment of luteal cells or membranes with LPA in vitro upregulates forskolin- as well as LH/GTP-stimulated adenylyl cyclase activity as reflected in a change of the Vmax of the enzyme. Although a PTX-sensitive G-protein has been proposed to participate in transmitting the inhibitory/stimulatory signals between LPA receptor and adenylyl cyclase, in luteal cells the observed LPA-induced upregulation of the enzyme activity was not abolished after PTX treatment, suggesting a different mode of action for these cells. The results from immunoblot analysis provided the evidence that type V adenylyl cyclase is present in luteal cells.

We have previously shown that phospholipase D is able to upregulate adenylyl cyclase in bovine luteal cells [13]. Consistent with the reported finding [3] that phospholipase D treatment of the cells activates putative endogenous LPA receptors we show here that [^{3}H]LPA could be cross-linked to a membrane protein of 38–40 kDa. Cold LPA displaced this binding in a dose-dependent manner indicating the specificity of the binding. A similar binding protein of about 40 kDa has been identified by photoaffinity labelling in various cell lines and in rat brain [5]. Although no LPA receptor has been purified so far, biochemical and molecular cloning data indicate the existence of multiple receptors for LPA-like mediators [6,25–28] widespread in different human tissues including the ovary [7].

In order to show functionality of LPA receptor in luteal cells, we took advantage of its ability to promote tyrosine

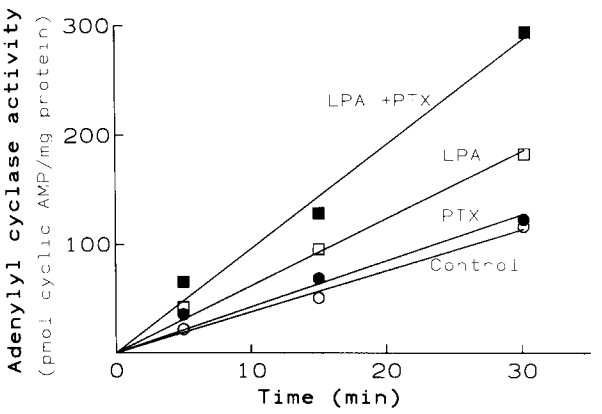


Fig. 5. Effects of PTX on LPA-induced sensitisation of adenylyl cyclase. The cells were treated for 180 min without (○, □) and with (●, ■) 10 ng/ml PTX and stimulated for further 15 min in the absence (○, ●) and presence (□, ■) of 100 μM LPA. The membrane fractions were used in the adenylyl cyclase assay performed in the presence of 100 ng/ml bovine LH and 10 μM GTP.

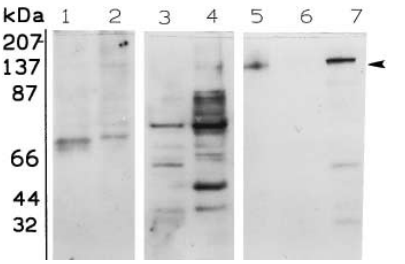


Fig. 6. Detection of adenylyl cyclase type V in bovine luteal cells by Western blotting. Luteal membranes were run on SDS-PAGE, blotted on to PVDF membrane and hybridised with different anti-adenylyl cyclase (AC) antisera. Lanes 1, 2 show blots hybridised with antibody against common AC (lane 1 cytosol, lane 2 membrane). Lanes 3, 4 show membrane (lane 4) and cytosolic (lane 3) fractions analysed with the antibody-recognising peptide sequence of the coding region of AC V. Lanes 5, 6 (membrane and cytosol respectively) and lane 7 (rat heart control) show blots immunoanalysed with antibody recognising the carboxy terminus of rat AC V.

phosphorylation in different cell systems in order to show that LPA induces tyrosine phosphorylation of a series of membrane bound proteins of 65–125 kDa in luteal cells. In other cell systems, LPA has been shown to phosphorylate proteins 110–130, 95, 65–75 and 42 kDa on tyrosine residues [29]. A non-receptor tyrosine kinase lying upstream of Rho has been suggested to transmit this LPA-elicited signals [2,3,30]. We show further that LPA sensitises the adenylyl cyclase in luteal cells by enhancing the Vmax of the enzyme. This agrees well with results published by Kreps et al. [11] and others [22], but is in contrast to reports where LPA has been shown to inhibit the cyclic AMP response [2,31]. Thus LPA has been reported to produce both stimulatory and inhibitory effects on forskolin- and hormone-stimulated adenylyl cyclase activity in diverse cells. Whether these effects of LPA are sensitive to PTX pre-treatment or not remains controversial. Unlike the Gi-mediated mitogenic response, the antimitogenic effect of LPA is not sensitive to PTX and is linked to an increase of cyclic AMP accumulation rather than decrease [32]. In 1321N1 cells and in human airway smooth muscle cells, however, LPA induces sensitisation of forskolin-stimulated cyclic AMP accumulation by a PTX-sensitive mechanism [11,12]. Interestingly however in the later report, PTX was unable to block the enhancement of isoproterenol-stimulated adenylyl cyclase by LPA [12]. Thus, the mechanism involved remains unclear.

In previous studies, presence of a PTX-mediated ADP-ribosylated protein has been reported [14] in bovine luteal cells [14]. Herrlich et al. [33] have identified two different PTX substrates in this cell system: Gi2 and Gi3, whereby only Gi2, but not Gi3 couples to LH receptor. PTX treatment augments LH-stimulated adenylyl cyclase activity in this cell system and partially abolished hCG-induced inositol formation [14,33]. However, we show here that the pre-treatment of bovine luteal cells with PTX did not abolish the LPA effects on the adenylyl cyclase activity, but even appeared to potentiate further the upregulation of the cyclase. Thus although some data show impaired function of the inhibitory Gi-function in LPA-stimulated cell systems, these possibilities are obviously not mutually exclusive for the LPA actions. In bovine luteal cells, the LH receptor has been shown [33] to couple simultaneously to both Gi2 and Gs, whereby αs stimulates adenylyl cyclase and $\beta\gamma^-$ subunits from both Gi and Gs

stimulate phospholipase C β2. In addition, dissociated αi can cause an inhibition of the cyclase thus damping the stimulatory αs action [33,34] indicating that the observed augmentation of LH-coupled adenylyl cyclase in PTX-treated cells results from the withdrawal of the Giα-mediated inhibition. Since LPA and PTX have additive effects in this cell system, we propose that the mechanism of LPA's action as far as adenylyl cyclase augmentation is concerned is distinct in these cells and independent of Gi2 coupling. It is not possible to exclude that LPA may couple to Gi3 in these cells thus regulating other pathways.

Cross-talk between adenylyl cyclase and other signalling cascades may occur via activation or inhibition of adenylyl cyclase by βγ⁻, αi subunits or by calcium released by another hormone not acting via Gs [35,36]. Although all mammalian cyclase isoforms are activated by Gsα, they differ dramatically in their response to other regulatory molecules with only a few common features [23,37]. Based on pharmacological characteristics, such as stimulation with Gsα, inhibition with Giα [33], additive effects of forskolin and GTP-analogs and positive modulation with protein kinase C [14], we can suggest that the adenylyl cyclase isoform being sensitised by LPA in luteal cells is presumably adenylyl cyclase type V. In fact we show that a protein band of 150 kDa is recognised by specific antibodies directed against adenylyl cyclase type V. These results certainly have to be confirmed on the molecular basis.

In conclusion, one can assume that the different extent and nature of the LPA-induced modulation of the cyclase activity depend upon specific subtypes of adenylyl cyclase and G-protein couplings present in a particular cell. It is possible that cells expressing the same cyclase isoforms may be differentially modulated through different G-protein couplings. Alternatively, expression of a given adenylyl cyclase isoform in a particular system may determine which regulatory pathway might be involved in the LPA regulation.

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