

# Differential effects of lysolipids on steroid synthesis in cells expressing endogenous LPA<sub>2</sub> receptor<sup>1</sup>

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**Abstract** Incubation of ovarian luteal cells with the bioactive lipid mediator lysophosphatidic acid (LPA) for 180 min abolishes gonadotropin-induced steroid production with no attenuation of the cyclic AMP accumulation. Treatment with the lysolipid also diminishes [<sup>14</sup>C]steroid production in cells preloaded with either [<sup>14</sup>C]cholesterol or [<sup>14</sup>C]acetate. Neither the expression of steroidogenic acute regulatory (StAR) protein nor in vitro steroid synthesis is affected in isolated mitochondrial fractions. The LPA-induced attenuation of steroid production occurs only in the mid-cycle corpus luteum and is associated with a transient endogenous expression of mRNA for the lysophosphatidic acid A<sub>2</sub> (LPA<sub>2</sub>) receptor (with no concomitant changes in the expression of LPA<sub>1</sub> receptor). Expression of LPA<sub>2</sub> is accompanied by LPA-induced sphingosine-1-phosphate (S1P) production. Because luteal cells, in the presence of the sphingosine kinase inhibitor dihydrosphingosine, can overcome the inhibitory effects of LPA on steroid synthesis, we suggest the possible requirement of intracellular S1P production. Interestingly, no LPA-induced inhibition of 8Br-cAMP-stimulated progesterone synthesis can be detected in Leydig tumor cell line MA10 cells expressing only LPA<sub>2</sub> receptor. Surprisingly, however, exogenous S1P inhibits agonist-stimulated progesterone in both cell types by inhibiting cyclic AMP accumulation, suggesting different mechanisms of action.—Budnik, L. T., and B. Brunswig-Spickenheier. Differential effects of lysolipids on steroid synthesis in cells expressing endogenous LPA<sub>2</sub> receptor. *J. Lipid Res.* 2005. 46: 930–941.

**Supplementary key words** lysophosphatidic acid • epidermal differentiating gene • sphingosine-1-phosphate • progesterone • steroidogenesis • ovary • luteal cells • corpus luteum • MA10 cells

Lysophosphatidic acid (LPA) is a lipid mediator with diverse growth factor-like biological activities accounting for much of the cellular proliferative effects of serum (1, 2). In addition, LPA affects such fundamental cellular functions as differentiation, survival, adhesion, and migration (1, 2). Generated from lysophospholipids, particularly from

lysophosphatidylcholine by lysophospholipase D, LPA is detected in several biological fluids, including blood, follicular fluid, and ovarian cancer ascites (3–5).

Bioactive lipid molecules signal through their cognate G-protein-coupled receptors, which belong to a growing family of lipid binding receptors previously named endothelial differentiation genes (Edgs) and renamed lysophosphatidic acid receptors A<sub>1</sub> (LPA<sub>1</sub>), A<sub>2</sub> (LPA<sub>2</sub>), and A<sub>3</sub> (LPA<sub>3</sub>) according to the International Union of Pharmacology nomenclature system (6). The main subgroups comprise LPA<sub>1–3</sub> (Edg2, -4, and -7) and S1P<sub>1–5</sub> (Edg1, -3, -5, -6, and -8), inheriting the receptors not only for LPA but also for its structural relative sphingosine-1-phosphate (S1P). Although most normal tissues express LPA<sub>1</sub> receptor, expression of LPA<sub>2</sub> has been shown only in tissues such as testis, thymus, spleen, stomach, and pancreas; other tissues, such as the ovary, showed little or no expression (7, 8). Some mammalian cells express only one LPA receptor form, whereas other cells spatially or temporally coexpress more than one receptor subtype (1).

The existence of various receptors raises the question of possible functional differences among the individual subtypes. Targeted disruption of LPA<sub>1</sub> resulted in 50% lethality in the perinatal period in mice (8, 9). Various functional defects, such as dysmorphisms, increased apoptosis, and hemorrhages, appeared in the remaining 50%, indicating the roles of LPA<sub>1</sub> in normal mammalian development. Mice with targeted deletion of LPA<sub>2</sub> do not show any obvious abnormalities (8); however, LPA<sub>2</sub> has been suggested to contribute to normal LPA-mediated signaling in some cell types (1). Research into neuronal development provides valuable information on the functional roles of individual LPA receptors (10–12). Both LPA<sub>1</sub> and

Abbreviations: Edg, epidermal differentiating gene; LPA, lysophosphatidic acid; LPA<sub>1</sub>, lysophosphatidic acid receptor A<sub>1</sub>; S1P, sphingosine-1-phosphate.

<sup>1</sup>This paper is dedicated to Prof. A. K. Mukhopadhyay, former Institute Director and our mentor.

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LPA<sub>2</sub> are implicated in brain formation during embryonic development (10). Studies based on data generated with neuronal cell lines overexpressing mouse receptor genes show both shared and distinct functions among the three LPA receptors (12). Because exogenous overexpression of LPA<sub>1</sub> in Schwann cells resulted in reduced apoptosis, and its overexpression in ovarian cancer cells induced apoptosis, this receptor subtype is implicated in cell survival (11). In LPA<sub>2</sub> receptor–dominant Jurkat cells, LPA evoked cell migration and suppression of IL-2 production, whereas in LPA<sub>1</sub>-dominant cells, LPA did not induce cell movement but did enhance IL-2 generation (13). The authors suggest that the effects of LPA in cells overexpressing LPA<sub>2</sub> or LPA<sub>1</sub> receptor only seem to mimic the effects observed in freshly isolated and activated helper T-cells, respectively (13). Information on the effects of various endogenously expressed LPA receptor subtypes in cell and organ physiology is rather sparse. Several malignancies show abnormal expression of various receptor subtypes (5). In epithelial ovarian cancer cells, which express high LPA<sub>2</sub> receptor (both the wild-type and the C-terminally-mutated EDG4 receptor mutant) levels and decreased LPA<sub>1</sub> levels, LPA induces mitogenic responses (14). Elevated levels of LPA found in ovarian cancer ascites are presumably responsible for elevated IL-8 production and contribute to the progression of this malignancy (14, 15).

In recent years, much attention has been focused on the role of LPA in tumor biology (5, 15), overshadowing studies linking this bioactive lipid to reproductive physiology (16–18). We showed previously that LPA elicits dramatic morphoregulatory effects on cultured luteal cells, steroid-producing cells originating from the ovarian corpus luteum (19). Assuming that the remodeling potential of LPA in the ovary is likely to have biological or pathological implications, we investigated the effect of LPA on steroid production in this cell system. The steroid hormone that most defines the physiological function of this transient reproductive organ is progesterone, which is necessary for the maintenance of pregnancy (20). When fertilization does not occur, the corpus luteum must stop producing progesterone, in order to allow the complex series of events that result in another ovulation. Progesterone is made from the precursor cholesterol delivered to the mitochondria, where the side chain is cleaved by the P450 side chain cleavage (scc) enzymes (21, 22). The cytoplasmic translocation of the esterified cholesterol precursor is dependent upon the cytoskeleton (21). Its shuttle from the outer to the inner mitochondrial membrane, facilitated by the steroidogenic acute regulatory (StAR) protein, is known as the rate-limiting step of the steroidogenesis (22, 23).

We show here that in luteal cells temporally coexpressing endogenous LPA<sub>2</sub> receptor during mid-cycle, LPA inhibits agonist-stimulated progesterone production at a step distal to the cyclic AMP formation and before the steroid synthesis in mitochondria. Interestingly, in progesterone-producing tumor cell line MA10 cells, which express only LPA<sub>2</sub> receptor, LPA did not attenuate steroid synthesis. In contrast to the effects of LPA, exogenous S1P inhibited agonist-stimulated progesterone synthesis and the cy-

clic AMP accumulation in both steroid-producing cells. The data suggest differential effects of the lysolipids on steroid synthesis and imply various mechanisms of action.

## MATERIALS AND METHODS

The sources of various chemicals were as follows: 1-oleoyl-LPA (1-oleoyl-sn-glycero-3-phosphate), 22(R)-hydroxycholesterol, and polyoxyethyl-cholesteryl sebacate (Cholesterol®), high-performance thin-layer chromatography (HPTLC) standards, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and DL-threo-dihydrosphingosine (DHS) protease inhibitors were from Sigma (Deisenhofen, Germany), [<sup>14</sup>C]cholesterol from NEN (Cologne, Germany), HPTLC plates from Whatman (Maidstone, UK), and TLC solvents from Merck (Darmstadt, Germany). Cyclic AMP, progesterone and pregnenolone assay reagents were from IHF (Hamburg, Germany). Forskolin (*Coleus forskohlii*) was provided by Calbiochem (Bad Soden, Germany). S1P was from Biomol (Hamburg, Germany). RNA purification kit was from Peqlab (Erlangen, Germany). SuperScript II reverse transcriptase, 100 bp size ladder standard, and low DNA mass ladder standard were from Invitrogen (Karlsruhe, Germany); dNTP, Oligo(dT)<sub>12–18</sub>, and [<sup>3</sup>H]choline chloride were from Amersham Pharmacia Biotech (Freiburg, Germany); BioTherm polymerase was from GeneCraft (Münster, Germany), QiaQuick gel extraction kit from Qiagen (Hilden, Germany), Abi Prism dye terminator sequencing ready reaction kit from Perkin Elmer (Branchburg, NJ), and oligonucleotides from MWG Biotech (Ebersberg, Germany). [<sup>14</sup>C]acetic acid sodium salt was from BioTrend (Cologne, Germany), and [U-<sup>14</sup>C]serine was from ICN Pharmaceuticals, Inc. (Irvine, CA). The polyclonal LPA<sub>1</sub>/edg2 antibody recognizing the p45 edg2 receptor in murine and p39-40 LPA<sub>1</sub> receptor in bovine tissues was from Upstate, Biomol (Hamburg, Germany). Western blot blocking substance, I-Block, was from Serva (Heidelberg, Germany) and the peroxidase-based detection system from Pierce (Perbio Science; Bonn, Germany). All secondary antibodies were from Jackson Immunochemicals (Dianova; Hamburg, Germany). Bovine dermal collagen was from Cellon (Strassen, Luxemburg). Bovine luteinizing hormone (LH) was a gift from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (Bethesda, MD), and Amicon Centricon filters were from Amicon-Millipore (Eschborn, Germany). The polyclonal anti-StAR antibodies were a gift from Dr. D. Stocco (Texas Tech University Health Sciences Center, Lubbock, Texas). All other reagents were obtained commercially and were of the highest purity.

### Cell isolation and culture procedures

The methods for the isolation and purification of bovine luteal cells have been published elsewhere (19). Briefly, the ovaries were obtained from the local abattoir, and for the isolation of the cells, corpora lutea from different luteal stages [early (LCp1), mid (LCp2), and late (LCp3)] (24) were used. The tissue was collagenase-dissociated and purified by centrifugation over percoll gradient. Purified cells were resuspended in Medium I (DMEM/HAM's F-12 medium, including 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5% heat-inactivated fetal calf serum), and the cell number was determined by counting on a hemocytometer. Cell viability, determined by trypan blue exclusion test, generally exceeded 90%. The cells were plated in duplicate on either 6-well plates (2,000,000 cells per well) or 50 ml polystyrene culture flasks (5,000,000 cells per flask) precoated with 0.3% Cellon collagen according to the procedure provided by the manufacturers. Cellon is prepared from pepsin-treated bovine dermis and consists of 99.8% pure collagen (95% type I

collagen and 5% type III collagen, with no degradation products). In some experiments described below (i.e., for the determination of steroids in culture medium), cells were grown on 24-well plates (250,000 cells per well). Cultures were grown in monolayers in Cellon-precoated dishes in Medium I. On the third day, the medium was replaced by fresh Medium II (DMEM/HAM's F-12 medium, including 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin supplemented with 5 µg/ml BSA, 5 µg/ml transferrin, 5 µg/ml insulin, and 5 ng/ml sodium selenite), and the cells were cultured further for 72 h before treatment. On day 7, 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 (Medium III). The conditioned medium was taken from the culture plates and used for the measurements of steroid accumulation as described below. The remaining adherent cells were fixed with methanol (5 min), stained (May-Grünwald staining method), and visualized using bright-field microscopy to monitor possible morphological or cell number changes during the incubation periods. The Leydig tumor cell line MA10 was routinely maintained in DMEM/HAM's F-12 Medium (1:1) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 7.5% horse serum plus 2.5% heat-inactivated fetal calf serum (Medium IV) in a humidified atmosphere of 5% CO<sub>2</sub>. The cell monolayers were grown to 80–100% confluency in this medium before being further cultured for 24 h in medium containing 0.5% serum and treated (i.e., stimulated or radiolabeled).

**Cell treatments.** Because LPA might be air and light sensitive, 5 mM stocks were made up in ethanol, and shortly before stimulation, ready-to-use samples were diluted/sonicated in serum-free medium. The cells were treated as indicated, the conditioned medium was aspirated, and, if not otherwise indicated (see below), the cells were centrifuged down (100 g for 10 min) at 4°C and washed twice, and the pellet was stored at –40°C.

### Preparation of mitochondrial fractions and analysis of StAR protein

For the preparation of mitochondrial fractions, the cells were resuspended in Tris buffer containing 10 mM Tris-HCl, pH 7.4, and 0.25 M sucrose plus 10 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml amastatin, and 1 mM phenylmethylsulfonic acid [protease inhibitors, (PIs)], including 0.25 M sucrose (Tris-sucrose buffer). The cells were homogenized using an all-glass Dounce-type homogenizer (25). The homogenate was centrifuged first at 600 g at 4°C for 15 min. The supernatant was then collected and recentrifuged at 10,000 g at 4°C for 15 min. This supernatant was removed, and the pellet was resuspended in Tris-sucrose buffer, centrifuged at 10,000 g at 4°C for 15 min, and diluted in Tris buffer. The individual fractions were filtered through Centricon Ultrafilter with 10 k-cutoff (Amicon-Millipore), and protein content was determined according to Bradford (26). For immunoanalysis of StAR protein expression in mitochondrial fractions, the proteins were separated electrophoretically on SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with I-Block (Tropix), and hybridized with polyclonal anti-StAR antibody diluted 1:5,000 as described earlier (27). The proteins expressed after stimulation were visualized using a peroxidase-based Luminol chemiluminescence system.

### In vitro steroidogenesis

The incubation of isolated mitochondria was carried out at 37°C as follows: 50 µg mitochondrial protein (prepared from treated and untreated cells) was incubated at 37°C in 25 mM Tris/HCl buffer, pH 7.4, (0.12 mM EDTA, 5 mM MgCl<sub>2</sub>, 0.1% BSA, 10 mM sodium-isocitrate, 0.15 M sucrose, and 10 mM potassium phosphate) with the addition of exogenous cholesterol in the form of

22(R)-hydroxycholesterol or membrane-permeable Cholesterol® as a substrate for pregnenolone production (10 µg/ml each). The incubation was carried out at 37°C for 90 min and was stopped by the addition of NaOH plus 0.14% Tween 20. After 15–18 h, 0.6 M H<sub>3</sub>PO<sub>4</sub> was added to avoid possible pH changes in pregnenolone ELISA. The amounts of pregnenolone produced were measured in a specific pregnenolone ELISA (IHF GmbH; Hamburg, Germany).

### Cholesterol metabolism

To measure cholesterol metabolism, the intracellular cholesterol pools were labeled for 24 h with 74 kBq/dish [<sup>14</sup>C]cholesterol (specific activity: 2.09 GBq/mmol) or with [<sup>3</sup>H]acetate (75 MBq/mmol) for 24 h in medium without serum (27). The cells were then washed and incubated in fresh medium for 3 h before stimulation with LPA as indicated. The cells were pelleted, and the radioactive lipids were extracted by the method of Bligh and Dyer (28). Labeled sterols were separated on silica gel HPTLC plates using the solvent system hexane-diethylether-acetic acid (130:30:1.5) as described earlier (27). Spots for cholesterol (R<sub>f</sub> 0.15–0.20), cholesterolone (R<sub>f</sub> 0.25–0.35), and cholesterol esters (R<sub>f</sub> 0.9–0.95) were identified from standards run in parallel and analyzed. To determine the incorporation of radioactivity into extracellular steroids, the conditioned medium was aspirated and extracted with 10 times sample volume of ethyl ether (29). The upper phase of ether extraction was evaporated to dryness, and the samples were dissolved in ethanol and applied to silica gel HPTLC plates. Steroids were measured using the solvent system benzene-ethyl acetate (4:1) according to Freeman and Ascoli (29). Authentic steroid standards were run on the same plates. The HPTLC plates were iodine-stained, and the samples were localized using a Berthold thin-layer radioactivity scanner (Wildbach, Germany). Authentic standards were run in parallel. The silica gel areas of interest were scraped and measured in a scintillation counter. For autoradiography, the plates were exposed at –80°C to Kodak βmax film.

### Steroid assays

The amounts of progesterone accumulated in medium were measured by the specific ELISA assay described earlier (18, 25). Briefly, the ELISA is a competitive double-antibody enzyme immunoassay and provides accurate measurements of progesterone in the range 0.14–34.02 ng/ml, corresponding to 1,701 pg/well. Within this range, the interassay coefficient of variation for the lowest standard was <15%. Relative cross-reactivity of the assay to structurally related compounds was estimated from the concentration required to yield 50% suppression of the biotinylated progesterone tracer. Cross-reactivity was minor with 11-deoxycorticosterone (9.5%), corticosterone (4.9%), and 17-α-hydroxyprogesterone (1.1%) and was negligible with an array of other steroids tested. (Full details of all components tested and the protocols can be obtained from Dr. M. Schumacher, IHF gGmbH, Hamburg, Germany.)

### Expression of LPA receptors

Total RNA was isolated by the method of Chomczynski and Sacchi (30) using the PeqGold RNA purification kit. The resulting RNA was dissolved in Tris-EDTA (TE) (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), and the concentration was determined by measuring the spectrophotometric absorbance at 260 nm. To estimate RNA integrity, an aliquot was electrophoretically separated on 1.5% agarose gel and visualized by staining with ethidium bromide. Following the manufacturer's instructions, first-strand cDNA was synthesized from 3 µg total RNA using SuperScript II reverse transcriptase, 0.5 mM each of deoxyribonucleoside triphosphate and 500 ng oligo(dT)<sub>12–18</sub> as primer. After comple-



tion of the reaction, 80  $\mu$ l of water was added to yield a final volume of 100  $\mu$ l. Two microliters of cDNA from theca, granulosa, luteal cells, and controls was used as template for PCR amplification of LPA receptors, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a valuable marker, as a housekeeping gene in ovarian cells (31, 32). In a total volume of 50  $\mu$ l, the PCR reaction mixture contained 2  $\mu$ l cDNA, 0.5 units BioTherm polymerase, 5  $\mu$ l 10 x BioTherm buffer, 1.5% Ficoll 400, 100 pmol of each primer, and 0.2 mM dNTP. In GAPDH, only 1  $\mu$ l cDNA and 50 pmol of each primer were used in the PCR reaction. Primers were designed on the basis of known sequences obtained from the National Center for Biotechnology Information data bank and were commercially synthesized. The following primers were employed for the PCR reaction: GAPDH: sense 5'-GTCTTCAC-CACCATGGAG-3', antisense 5'-GTCATGGATGACCTTGGC-3' (yielding a fragment with an expected size of 198 bp); edg-2 (LPA<sub>1</sub>): sense 5'-ATCTTTGGCTATGTTCGCCA-3', antisense 5'-TTGCTGTGAACTCCAGCCA-3' (yielding a fragment with an expected size of 394 bp); LPA<sub>2</sub>: sense 5'-TGGCCTACCTCTTC-CTCATGTTCCA-3', antisense 5'-GGGTCCAGCACACCACAAA-TGCC-3' (yielding a fragment with an expected size of 516 bp); and LPA<sub>3</sub>: sense 5'-AGTGTCACTATGACAAGC-3', antisense 5'-GAGATGTTGCAGAGGC-3' (yielding a fragment with an expected size of 513 bp). PCR conditions were as follows: after a 2 min denaturation step at 95°C, samples were subjected to 30 cycles at 95°C for 1 min, 48°C [edg-2 (LPA<sub>1</sub>)], 59°C LPA<sub>2</sub>, or 53°C for 1 min LPA<sub>1</sub> and GAPDH, respectively) and 72°C for 1 min, followed by an additional elongation step at 72°C for 10 min (25). As a negative control, cDNA was replaced by water in the reaction mixture. The PCR products were separated on 1.5% agarose gel, and the length of the fragments was determined by comparison with a 100 bp size ladder standard. To verify the identity of GAPDH and LPA<sub>1</sub>, bands were cut from the gel and isolated with a QiaQuick gel extraction kit. The purified DNA fragments were dissolved in TE and quantified spectrophotometrically and by comparison to a low DNA mass ladder standard. A 100 ng aliquot of the respective PCR products was amplified using Abi Prism Dye Terminator Se-

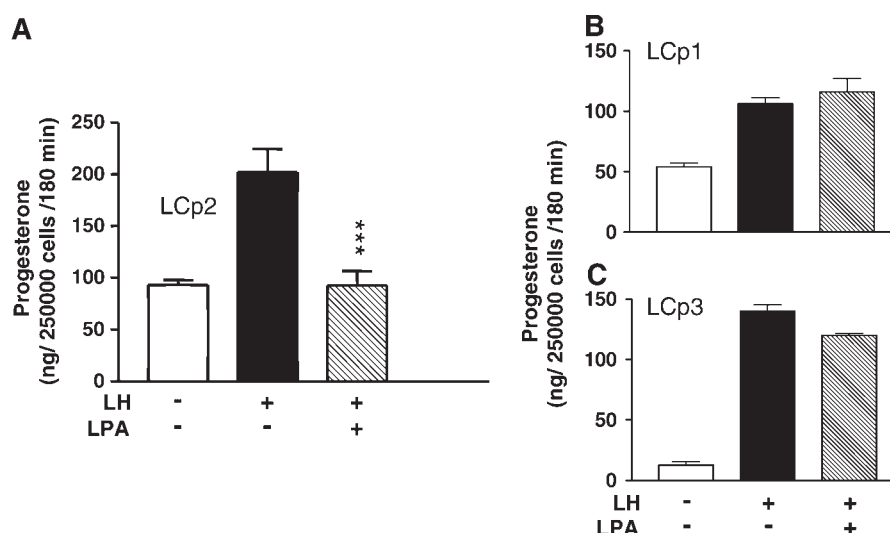
quencing Ready Reaction Kit according to the manufacturer's manual and sequenced commercially on an Abi Prism 377 sequencer. The sequences of the PCR fragments showed 100% identity to published sequences (bovine skeletal muscle or mouse testis).

### S1P synthesis

To measure the sphingolipid metabolites, the confluent cell monolayers were labeled for 24 h with [<sup>14</sup>C]serine (specific activity: 5.66 GBq/mmol), and the cells were rinsed three times with fresh medium and stimulated with LPA for the indicated time periods. The cells were scraped from the dish and were subjected to lipid extraction as described above. The lipids present in the organic phase were saponified with 0.1 M methanolic KOH for 1 h at 37°C (33) to remove glycerophospholipids. The alkali stable sphingolipids were resolved on HPTLC plates using sequential two-solvent analysis: chloroform-methanol-ammonia (65:25:5) for 10 cm, followed by benzene-ethyl acetate (65:35) to the top of the plate. Standards for S1P, sphingosine, ceramide, and ceramide-phosphate were separated on the same plate. The samples and standards were monitored with a radioactivity scanner (Berthold Systems). The TLC areas corresponding to the lanes [<sup>14</sup>C]S1P ( $R_f$  0.13–0.18) were scraped from the plates, placed in scintillation vials, extracted with methanol-HCl (150:1), and counted in a beta counter after the addition of 9 ml toluene-based scintillation fluid. In control experiments, the cells were loaded with [<sup>14</sup>C]stearic acid (specific activity: 2.04 GBq/mmol) and [<sup>3</sup>H]choline (data not shown). In parallel experiments (data not shown), the possible changes in the amounts of the sphingosine and N-acylated sphingosine (ceramide) were examined either on the same plate or in additional control experiments using HPTLC plates activated by prerunning with acetone and resolved using a sequential two-solvent system chloroform-methanol-acetic acid-water (25:15:4:1.5) followed by chloroform-methanol-acetic acid (65:2.5:4).

### Endogenous expression of LPA<sub>1</sub> receptor protein

Luteal cells and MA10 cells (or control cells) were harvested by gently scraping with a rubber policeman into ice-cold isotonic



**Fig. 1.** Inhibition of LH-stimulated steroid production by lysophosphatidic acid (LPA) in luteal cells. Cultured luteal cells from early (LCp1), mid-cycle (LCp2), and late (LCp3) luteal phases were stimulated for 180 min with medium only (no addition) or with 100 ng/ml luteinizing hormone (LH) in the presence or absence of 1  $\mu$ M LPA. The medium was aspirated, and the amounts of the steroid produced during the stimulation were measured using a specific progesterone ELISA and analyzed with statistical software as described in Materials and Methods. The data are shown as mean  $\pm$  SD ( $n = 8$  for A;  $n = 3$  for B and C). Comparisons were made between LH and LH plus LPA; \*\*\*  $P < 0.001$ .

sucrose buffer A containing: 10 mM Tris-HCl, pH 7.4, and 0.25 M sucrose plus 10  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1  $\mu$ g/ml amastatin, and 1 mM phenylmethylsulfonyl acid (PI) before gentle homogenization as described previously (25). The resulting homogenate was first centrifuged at 100  $g$  for 15 min to yield pellet and supernatant. The supernatant was recentrifuged at 100,000  $g$  for 60 min to yield particulate membrane fractions and the supernatant (cytosolic fractions). Subfractions were filtered through Centricon Ultrafilters with 10k-cutoff, and protein content was determined according to Bradford (26). Equal amounts of proteins were separated by SDS-PAGE (80  $\mu$ g), transferred to PVDF membranes, and stained with Ponceau S. Blots were blocked with I-Block blocking substance and incubated overnight at 4°C with polyclonal antibodies recognizing the edg2/LPA<sub>1</sub> receptor (1:1,000). As control, blots were rehybridized with antibodies directed against  $\beta$ -actin (1:5,000). After incubation with the peroxidase-conjugated affinity-purified secondary antibody (1:4,000), the immune complexes were detected using luminol-based chemiluminescence systems. Additionally, control blots were run with nonimmune serum and the secondary antibodies.

### Data analyses

Each experiment was performed at least three times using the ovaries from different animals ( $n = 3$  or  $n = 5$ ). Blots and films were analyzed densitometrically using NIH Scion Image Program (Scion Corporation; Frederick, MD) and were quantified with the computer-assisted analysis program using both threshold and density slicing modes to provide area and density measurements of the gray-scale images. The values were compared with control bands (i.e.,  $\beta$ -actin or GAPDH). Integrated optical density bands were analyzed statistically as described below. The data are presented as mean values ( $\pm$ SD) from three or more experiments (as indicated). Additionally, individual representative data sets are shown. The EC<sub>50</sub> and the statistical analysis of the data presented were performed using the computer program Instat Prism Software from GraphPad Software, Inc. (San Diego, CA). The first tests were performed using standard  $t$ -tests, and further analysis was carried out using repeated measures one-way ANOVA and Dunnett's post test. The probability values ( $P$ ) of  $>0.05$  were considered as not significant (NS), the values 0.01–0.05 as significant, 0.001–0.01 as very significant, and  $<0.001$  as extremely significant.

## RESULTS

### LPA inhibited agonist-activated steroid synthesis

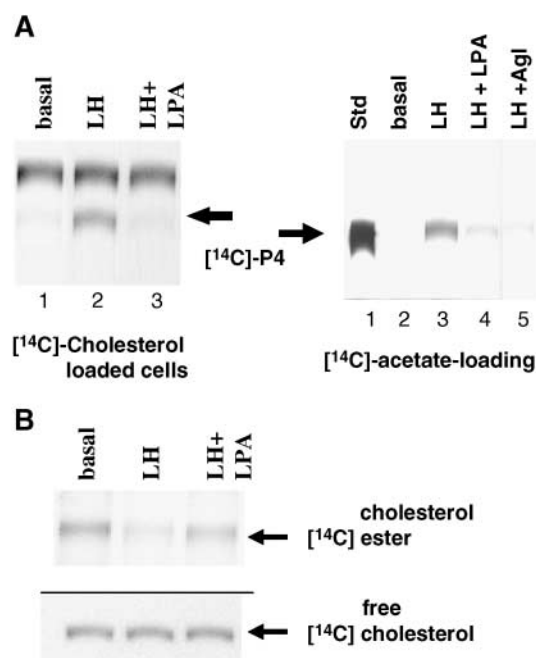
Incubation of ovarian mid-cycle luteal cells (LCp2) with LPA for 180 min resulted in a significant inhibition of agonist-stimulated steroid production (Fig. 1A). Ten nanomolar LPA led to a reduction of LH-induced progesterone production, reaching a maximal inhibitory effect with 1  $\mu$ M LPA, with no further inhibition for doses up to 100  $\mu$ M (data not shown). The attenuation of the steroid synthesis may not appear to be very dramatic, but a constant (40–90%) inhibition could be observed at all LH concentrations tested (data not shown). The calculated EC<sub>50</sub> doses were 100 nM. The effect appeared to be specific, inasmuch as incubation with LPA up to 100  $\mu$ M did not attenuate the basal progesterone synthesis; instead, a slight stimulation could be monitored with LPA concentrations up to 50  $\mu$ M (data not shown).

When the luteal cells from early luteal phase (LCp1)

were used (Fig. 1B), no inhibition of the steroid synthesis was monitored. When cells from late luteal phase (LCp3) were stimulated with the gonadotropin in the presence of LPA (Fig. 1C), only a minor inhibition was observed (10–20%).

### LPA inhibited [<sup>14</sup>C]progesterone synthesis in [<sup>14</sup>C]cholesterol-loaded cells

Several rate-limiting steps define the steroidogenic pathway (22). The availability of the cholesterol is a major prerequisite for steroidogenesis. When luteal cells were loaded with [<sup>14</sup>C]cholesterol, an LH-induced accumulation of [<sup>14</sup>C]progesterone was observed (Fig. 2A). Treatment with LPA inhibited the synthesis of the radioactive steroid. If, instead of [<sup>14</sup>C]cholesterol, the cells were loaded with [<sup>14</sup>C]acetate to initiate de novo cholesterol synthesis, a similar inhibition of [<sup>14</sup>C]progesterone could be monitored (Fig. 2A, right figure). Cells treated with aminoglutethimide, an inhibitor of P450<sub>scc</sub>, were used as an internal negative control, and radioactive progesterone was used as a



**Fig. 2.** Inhibition of LH-stimulated [<sup>14</sup>C]progesterone production in [<sup>14</sup>C]cholesterol-loaded cells. LCp2 cells loaded with [<sup>14</sup>C]cholesterol or [<sup>14</sup>C]acetate were stimulated with either medium only (basal), LH (100 ng/ml), or LH plus LPA (1  $\mu$ M) for 180 min (A, B). The cells were pelleted, and radioactive lipids were extracted and separated on silica high-performance thin-layer chromatography (HPTLC) plates using the solvent system hexane-diethylether-acetic acid (130:30:1.5) to identify cholesterol ( $R_f$  0.15–0.20), cholesterolone ( $R_f$  0.25–0.35), and cholesteryl esters ( $R_f$  0.9–0.95). The conditioned medium was extracted with diethyl ether, the upper phase was evaporated to dryness, the samples were dissolved in ethanol, and [<sup>14</sup>C]progesterone ([<sup>14</sup>C]P4) was separated on the HPTLC plates using the solvent system benzene-ethyl acetate (4:1). Authentic standards were run on each plate. The control lane (LH + AgI) shows cells treated with 100  $\mu$ M aminoglutethimide inhibiting the mitochondrial side-chain cleavage activity. For the autoradiography, the HPTLC plates were exposed at  $-80^\circ\text{C}$  to Kodak  $\beta$ max film.

marker (Fig. 2A, Std). Parallel to its effects on the [ $^{14}\text{C}$ ]progesterone synthesis, the LPA-induced attenuation of agonist-induced decrease in the amounts of the cholesterol ester was monitored (Fig. 2B). No effect on the level of free cholesterol was observed (Fig. 2B).

#### LPA did not affect either in vitro steroid synthesis or the expression of the StAR protein in isolated mitochondria fractions

To examine further whether LPA treatment might directly affect steroid production in vitro, we stimulated the cells with or without LPA and isolated pure mitochondria fractions. When either membrane-permeable Cholesterol<sup>®</sup> or 22(R)-hydroxycholesterol was used as a substrate, the mitochondria were able to synthesize pregnenolone in vitro. Using either of the membrane-permeable cholesterol forms, we observed a linear reaction dependent on the incubation time and the amounts of the protein used (data not shown). However, no inhibition of the steroidogenesis could be monitored when mitochondria from LPA-treated cells were used, as compared with untreated cells (Fig. 3A). In fact, a slight—although not significant (10–15%)—elevation of the steroid amounts was observed.

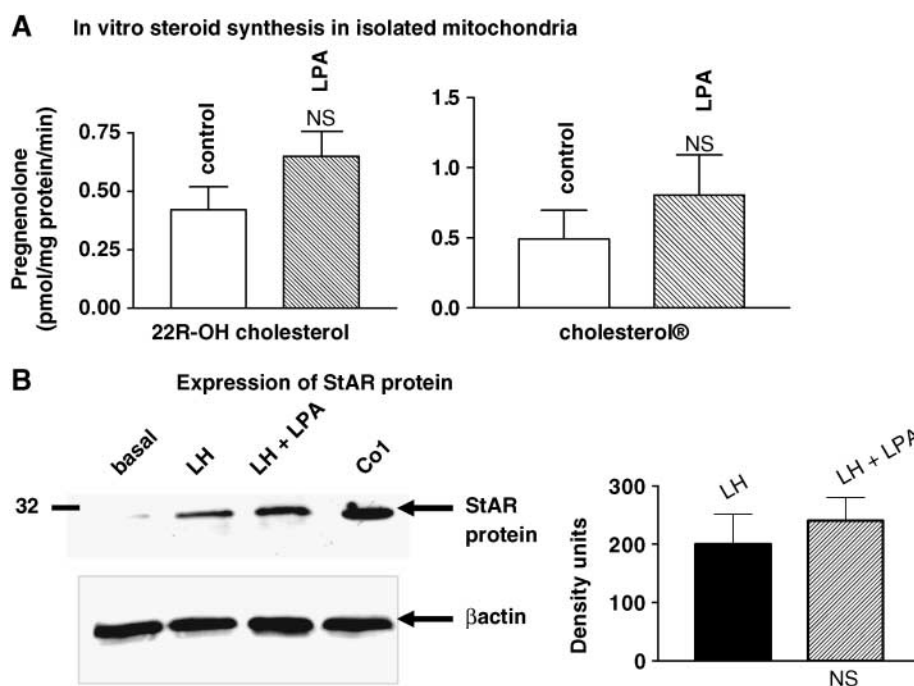
The transfer of the cholesterol from the outer mito-

chondrial membrane through the intermembrane space is facilitated by a protein mediator, the steroidogenic acute regulatory protein, StAR (22). The 30 kDa StAR protein, expressed in mitochondria of hormone-stimulated steroid-producing cells, may impact the cholesterol delivery within the mitochondria (22–23). The examination of the mitochondrial StAR protein expression in luteal cells treated with either LH or LH plus LPA showed no dramatic changes (statistically not significant), indicating a distal point of action (Fig. 3B).

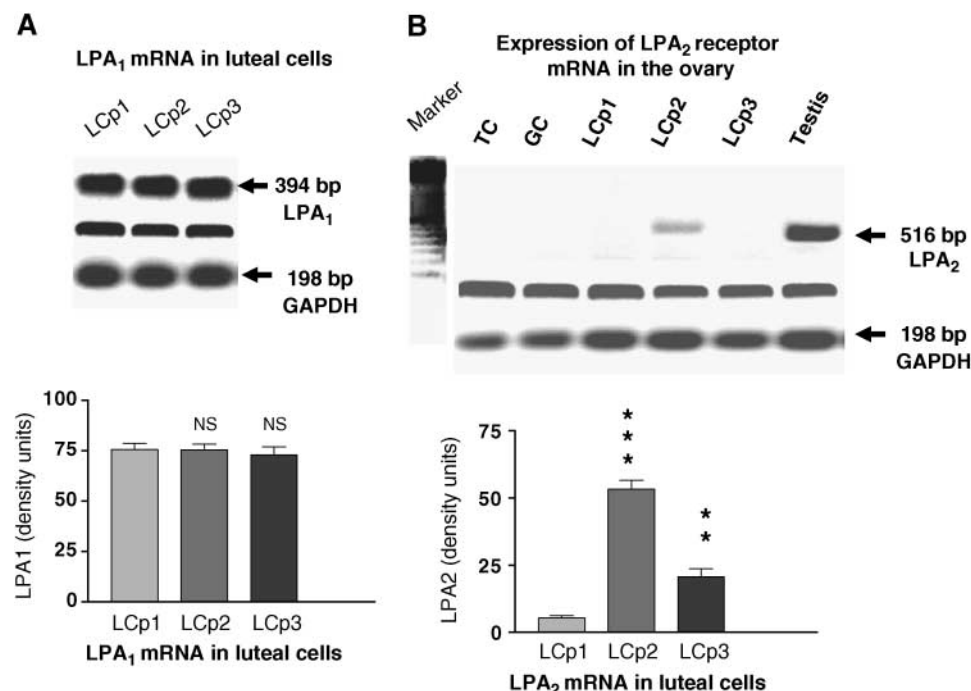
#### The transient expression of the LPA<sub>2</sub> receptor in mid-cycle corpus luteum

We showed previously that virtually all steroidogenic cell compartments express LPA<sub>1</sub> receptor (25). In the present study, we carefully analyzed the three stages of corpus luteum maturation to evaluate possible changes in the receptor expression. As shown in Fig. 4A, we could not detect any significant changes in the expression of the LPA<sub>1</sub> receptor in luteal cells at various stages of the corpus luteum development. Western blot analysis also showed no detectable changes in LPA<sub>1</sub> protein level (data not shown).

When examining the expression of the LPA<sub>2</sub> receptor, we could—surprisingly—observe a transient expression of



**Fig. 3.** LPA did not affect in vitro steroid synthesis or the expression of the StAR protein in isolated mitochondria fractions. **A:** Isolated mitochondria from control cells or cells stimulated with LPA for 180 min were used to settle in vitro steroid assay using two different membrane-permeable cholesterol forms (10  $\mu\text{g}/\text{ml}$  each), 22(R)-hydroxycholesterol and polyoxyethanyl-cholesteryl sebacate (Cholesterol<sup>®</sup>) as substrates for mitochondrial pregnenolone synthesis. The incubation was carried out at 37°C as described in Materials and Methods. The amounts of the pregnenolone were measured by a specific ELISA. **B:** For the immunoanalysis of the inducible StAR protein, the mitochondrial fractions from cells treated for 180 min with either medium only, LH, or LH plus LPA were separated on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The expression of the StAR protein was measured using a specific anti-StAR antibody (1:5,000). The last lane (Co1) shows the mitochondrial fractions prepared from 8Br-cAMP-stimulated MA10 cells (used in control experiments). The blots were stripped and rehybridized with  $\beta$ -actin. Comparisons were made between LH and LH plus LPA. Error bars indicate SD.



**Fig. 4.** Transient endogenous expression of LPA<sub>2</sub> receptors in mid-cycle corpus luteum. A: cDNA from luteal cells isolated from various corpus luteum stages (LCp1, LCp2, LCp3) was used to analyze the presence of 394 bp LPA<sub>1</sub> fragment, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. The analysis was performed using samples obtained from three different cell preparations. B: cDNA from various theca (TC), granulosa (GC), luteal cells (LC), and from control tissues (mouse testis) was used as a template for PCR amplification of LPA<sub>2</sub>, with GAPDH as a housekeeping gene. Primer design and PCR conditions are described in Materials and Methods. The arrows show specific 394 bp LPA<sub>1</sub>, 516 bp LPA<sub>2</sub>, and 198 bp GAPDH fragments. The expression of the endogenous LPA<sub>2</sub> message was analyzed using mRNA from three independent cell preparations and from three different corpus luteum stages each [early (LCp1), mid (LCp2), and late (LCp3) luteal phase]. Comparisons were made between LCp1, LCp2, and LCp3: \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; not significant (NS),  $P > 0.05$ . Error bars indicate SD.

endogenous LPA<sub>2</sub> mRNA message in LCp2 cells (Fig. 4B). Could the co-appearance of the LPA<sub>2</sub> receptor in fact link LPA signaling to the inhibition of the steroid synthesis?

#### LPA stimulated intracellular S1P accumulation in LCp2 cells

In parallel experiments, we also observed that the stimulation of LCp2 cells with LPA resulted in a rapid intracellular [<sup>14</sup>C]S1P accumulation (Fig. 5). The pretreatment with 10  $\mu$ M DL-threo-DHS blocked the intracellular S1P accumulation by 90%. DHS was shown to inhibit the sphingosine kinase activity in other cell systems in the range 8–18  $\mu$ M (34, 35). There was no LPA effect on either sphingosine, N-acetylated sphingosine, or ceramide-1-phosphate contents under the experimental conditions used (data not shown).

#### Inhibition of sphingosine kinase-mediated intracellular S1P synthesis attenuates the LPA-induced effects on steroid production

Interestingly, addition of DHS reversed nearly completely (80–90%) the inhibitory effects of LPA on steroidogenesis (Fig. 6). Moreover, S1P itself mimicked the effects of LPA and inhibited LH-stimulated progesterone production (Fig. 6).

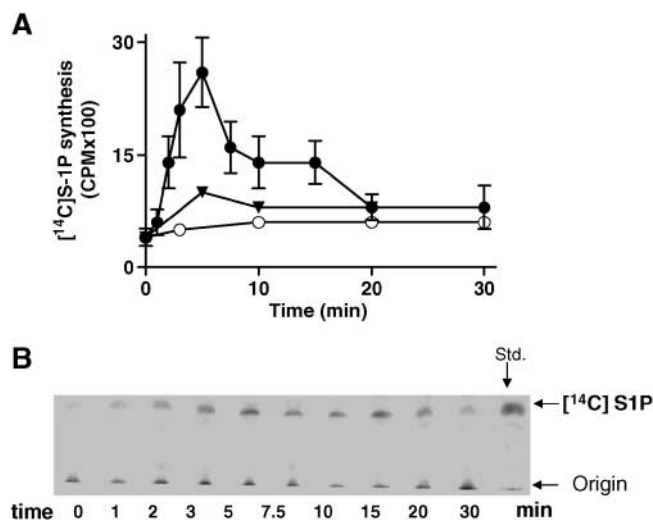
#### LPA inhibits agonist-stimulated steroidogenesis in LCp2 cells, but not in MA10 cells, whereas S1P inhibits progesterone synthesis in both cell types

We next evaluated the influence of the exogenous lysolipids LPA and S1P in a tumor cell line, MA10, known to synthesize progesterone. As shown in Fig. 7A, LPA did not inhibit the agonist-stimulated steroid synthesis in these cells. In contrast, 1  $\mu$ M S1P inhibited 8Br-cAMP-stimulated progesterone synthesis in both MA10 cells and LCp2 cells (Fig. 7A, B). Whereas the addition of LPA concomitantly with S1P resulted in a complete inhibition of the steroid synthesis in LCp2 cells (Fig. 7B), there was no additional influence of LPA on the effects of S1P in MA10 cells (Fig. 7A).

#### LPA did not affect agonist-stimulated cyclic AMP accumulation in either luteal (LCp2) or MA10 cells

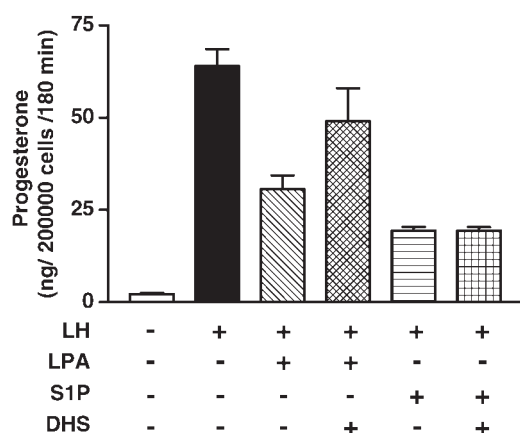
When the amounts of accumulated cyclic AMP were measured, it became evident that LPA did not abolish agonist-stimulated cyclic AMP formation in either progesterone-synthesizing cell type (Fig. 8A, B). The addition of *Coleus forskohlii* (forskolin), which stimulated the catalytic unit of the adenyl cyclase, induced both cyclic AMP formation and progesterone production. In luteal cells (LCp2) treated with forskolin (at concentrations up to 50  $\mu$ M), LPA inhib-



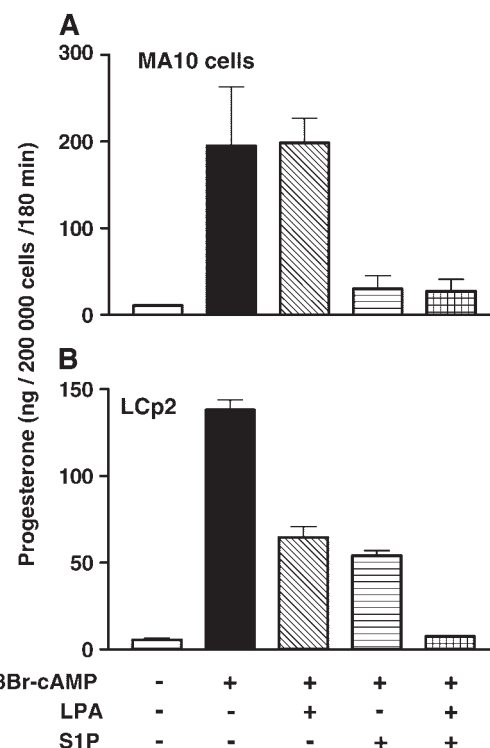


**Fig. 5.** LPA stimulates intracellular sphingosine-1-phosphate (S1P) synthesis. **A:** Cultured luteal cells loaded with [ $^{14}\text{C}$ ]serine were pretreated for 10 min with medium only (open and closed circles) or with 10  $\mu\text{M}$  sphingosine kinase inhibitor DL-threo-dihydro-sphingosine (DHS) (triangles) and were further stimulated for various time periods without any addition (open circles), or with 1  $\mu\text{M}$  LPA alone (closed circles) or with LPA in the presence of DHS (triangles). The amounts of the synthesized [ $^{14}\text{C}$ ]S1P were measured in alkali stable lipid extract resolved on the HPTLC plates using sequential two-solvent analysis: chloroform-methanol-ammonia (65:25:5) for 10 cm followed by benzene-ethyl acetate (65:35) to the top of the plate. Standards for S1P ( $R_f$  0.13–0.18) were separated on the same plate. The samples were localized using a TLC scanner and counted in a beta-counter as described in Materials and Methods. **B:** A representative autoradiogram. Error bars indicate SD.

ited also the steroid synthesis stimulated by this agonist, providing additional support for the mechanism distal to LH binding and beyond the cyclic AMP accumulation (data not shown). In contrast, exogenous S1P inhibited cyclic AMP accumulation in both LCp2 and MA10 cells (Fig. 8A, B).



**Fig. 6.** Inhibition of the sphingosine kinase attenuates the LPA-induced effects on steroid synthesis in LCp2 cells. The LCp2 cells were pretreated for 10 min with or without 10  $\mu\text{M}$  DHS and were stimulated further with medium only (basal), with 100 ng/ml LH, with LH plus 1  $\mu\text{M}$  LPA, or with LH plus 1  $\mu\text{M}$  S1P in the presence or absence of DHS. The amounts of the synthesized progesterone were analyzed as described in the legend to Fig. 1. Error bars indicate SD.



**Fig. 7.** LPA inhibits agonist-induced steroidogenesis in LCp2 cells, but not in MA10 cells, whereas S1P inhibits progesterone synthesis in both cell types. The MA10 cells (**A**) or LCp2 cells (**B**) were stimulated with medium only (basal), with 1 mM 8Br-cAMP, or with 8Br-cAMP in the presence of either 1  $\mu\text{M}$  LPA, 1  $\mu\text{M}$  S1P, or LPA plus S1P. The amounts of the synthesized progesterone were analyzed as described in the legend to Fig. 1. Error bars indicate SD.

#### MA10 cells express LPA<sub>2</sub> receptor, but not LPA<sub>1</sub>, whereas LCp2 cells express both LPA receptor types

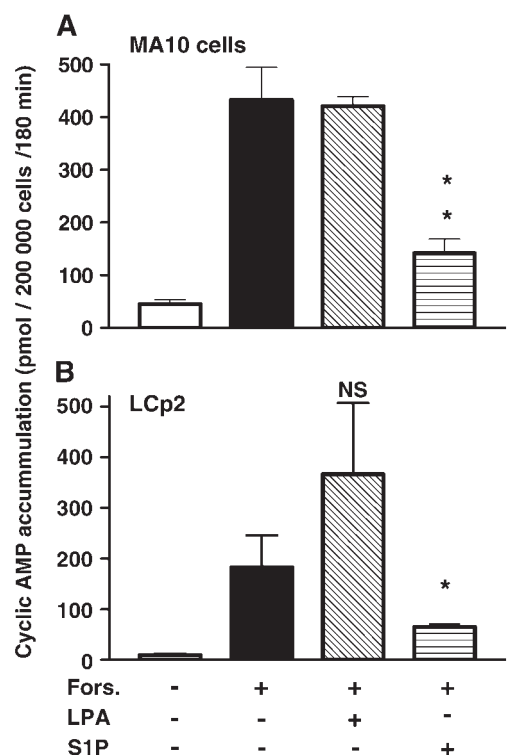
Because LPA apparently had a different effect on each cell type, it became necessary to compare the LPA receptor expression in the two steroidogenic cells. Surprisingly, the MA10 cells expressed only LPA<sub>2</sub> receptor (Fig. 9). Control data (Fig. 10) showed that although all ovarian cells (luteal LCp1, LCp2, LCp3, and theca) showed endogenous expression of LPA<sub>1</sub> receptor protein, no such signal could be detected when equal amounts of MA10 cell membrane protein were blotted simultaneously.

Neither LCp2 cells nor MA10 cells expressed LPA<sub>3</sub> (or showed an extremely weak signal, as shown in Fig. 9). Thus, based on the lack of the mRNA for LPA<sub>1</sub>, the MA10 cells appear to provide a useful model to study the effects of a native LPA<sub>2</sub>. However, whereas the MA10 cells did not show any LPA<sub>1</sub> message, the LCp2 cells transiently expressed the LPA<sub>2</sub> mRNA, keeping the LPA<sub>1</sub> message at a constant level.

#### DISCUSSION

We studied here LPA receptor-mediated effects on steroid synthesis and observed that LPA inhibited gonadotropin, LH-induced steroidogenesis in ovarian luteal cells.





**Fig. 8.** S1P, but not LPA, inhibits forskolin-stimulated cyclic AMP accumulation. Cultured MA10 cells (A) or LCp2 cells (B) were stimulated with medium only (basal), with 25  $\mu$ M forskolin (Fors.) and with forskolin in the presence of either LPA (1  $\mu$ M) or S1P (1  $\mu$ M). The amounts of the total cyclic AMP accumulated were measured with specific ELISA assays as described in Materials and Methods. Comparisons were made between forskolin only, forskolin plus LPA, and forskolin plus S1P. \*  $P < 0.1$ ; \*\*  $P < 0.01$ ; NS,  $P > 0.05$ ;  $n = 3$ . Error bars indicate SD.

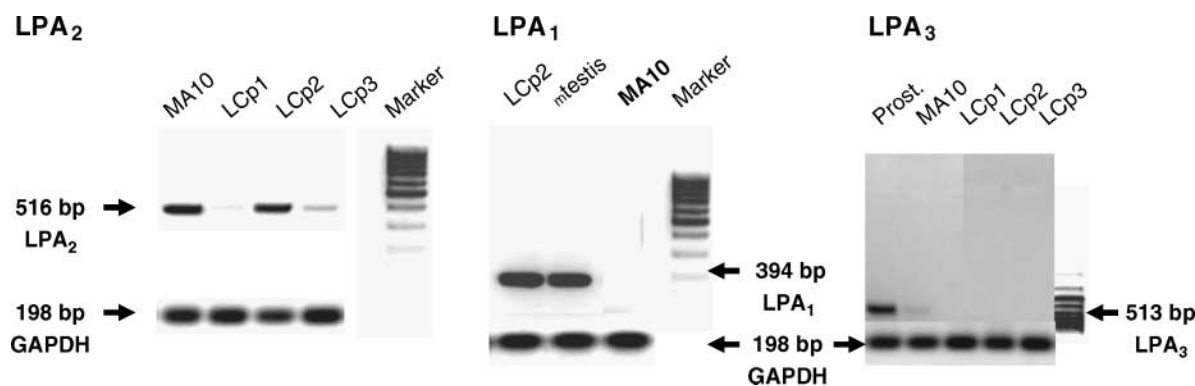
The attenuation of the steroid production occurred in the mid-cycle corpus luteum only (LCp2 cells) and was associated with transient endogenous expression of mRNA for the LPA<sub>2</sub> receptor (with constant LPA<sub>1</sub> expression throughout the cycle). In the presence of sphingosine kinase inhibitor, the LCp2 cells could partially overcome the inhibitory effects of LPA. Because LPA stimulated intracellular S1P production in LCp2 cells, we suggest that inhibition of the steroid synthesis process may possibly require LPA-induced intracellular S1P production. On the other hand, LPA does not inhibit progesterone synthesis in MA10 cells, a cell line which expresses LPA<sub>2</sub>, but no LPA<sub>1</sub>. It appears that an aberrant LPA<sub>2</sub>/LPA<sub>1</sub> ratio during the mid-cycle impacts the LPA signaling in LCp2 cells, leading to the temporary attenuation of steroidogenesis. In contrast, exogenous S1P inhibited steroid synthesis in both cell types in a cyclic AMP-dependent manner. The data suggest that although both lysolipids attenuate steroidogenesis, their mechanisms of action differ.

Luteal cells possess specific LPA binding sites (36), and all steroidogenic cells of the ovary, including the luteal cells, express LPA<sub>1</sub> receptor mRNA and protein (25). Although the expression of the LPA<sub>1</sub> receptor was not altered in the course of the cycle, we could monitor a transient endogenous expression of an additional LPA receptor,

LPA<sub>2</sub>, associated with the mid-cycle (LCp2). This was surprising, because the presence of LPA<sub>2</sub> was shown in ovarian carcinoma only (37); its expression under physiological conditions has not as yet been documented. In fact, because the related carboxyl-terminus mutant of LPA<sub>2</sub>, termed edg4, was isolated from neoplasm, the expression of LPA<sub>2</sub> was initially implicated in carcinogenesis (1, 38). More recent data show expression of LPA<sub>2</sub> also during neuronal differentiation and during immune responses (1). Interestingly, the LPA<sub>2</sub>/edg4 receptor coupled the LPA signal to S1P synthesis in SH-SY5Y neuroblastoma, and RH7777 hepatoma cells (39, 40). This novel pathway associated with endogenous LPA<sub>2</sub>/edg4 receptor activation could explain the complex relationship between receptor activation and the resulting stimulation of S1P synthesis (39, 40).

The mechanisms and specificity by which the individual LPA receptors mediate the LPA actions are still poorly understood. The current study showed that the endogenous expression of LPA<sub>2</sub> correlates with LPA-induced inhibition of steroid production. It remains to be clarified whether the LPA<sub>2</sub> per se couples the LPA signaling to the steroid pathway or whether the concomitant presence of the LPA<sub>1</sub> and LPA<sub>2</sub> receptors (altering the LPA<sub>2</sub>/LPA<sub>1</sub> ratio) was responsible for the observed effects. Similar aberrant expression of the LPA receptors was observed during malignant transformation in human colorectal cancer (41). Immune cell systems showed as well a highly flexible LPA receptor expression pattern (42). Although no subset of native T cells expressed LPA<sub>1</sub> receptor alone, the activated cells expressed LPA<sub>2</sub> and LPA<sub>1</sub> concomitantly, showing opposing effects of the LPA signal transduced by either LPA<sub>2</sub> or LPA<sub>1</sub> receptor (42). In ovarian cancer cells, LPA<sub>2</sub> and LPA<sub>1</sub> receptors (both native and transfected) showed suppressive influence on individual LPA-induced signal transduction pathways (43, 44). Not only may the relative expression of LPA receptors impact LPA signaling, but also the individual LPA receptors may couple to various signal transduction pathways, leading to the differential regulation of various G-protein couplings and the functional activation of small RhoGTPases. LPA<sub>2</sub> receptor linked LPA to IL-6 and IL-8 production and coupled to TRIP6/ZRP-1 protein (thyroid receptor interacting protein/zyxin-related protein) in ovarian cancer cells (45, 46). In Leydig tumor cell (MA10 cells) expressing LPA<sub>2</sub> receptor only (but no LPA<sub>1</sub>), S1P, but not the LPA, induced the inhibition of steroidogenesis. This may indicate that the aberrant LPA<sub>2</sub>/LPA<sub>1</sub> ratio altered the LPA responses in LCp2 cells, resulting in inhibition of progesterone production, rather than a single transient expression of the LPA<sub>2</sub> type. Interestingly, a similar transient expression of LPA<sub>2</sub> is also seen in corpus luteum isolated from primate ovary (data not shown).

S1P is itself an important lipid mediator, implicated in many biological processes, and, unlike LPA, can exert its action not only through G-protein-coupled receptor, but also as an intracellular second messenger that regulates environmental stress responses and survival (47, 48). Intracellular S1P acts as an autocrine signal, regulating the actin cytoskeleton during neuronal Schwann cell and neo-



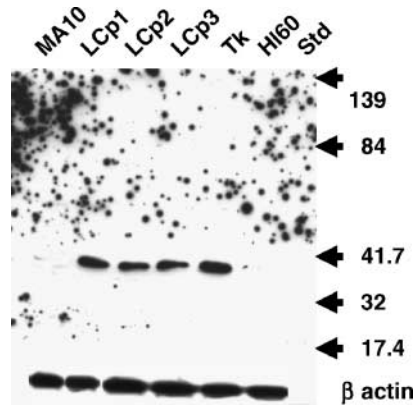
**Fig. 9.** MA10 cells express LPA<sub>2</sub> receptor and trace amounts of LPA<sub>3</sub>, but no LPA<sub>1</sub> receptor. CDNAs, either from luteal cells isolated from various corpus luteum stages (LCp1, LCp2, LCp3), from MA10 cells from whole testis (<sub>m</sub>testis), or from prostate tissue (Prost.) were used to analyze the presence of 394 bp LPA<sub>1</sub> fragment, the specific 516 bp LPA<sub>2</sub> fragment, 513 bp LPA<sub>3</sub>, with GAPDH as the housekeeping gene, as shown in the legend to Fig. 4.

natal cardiac myocyte development (49, 50). The effect of SIP on steroid synthesis has not as yet been investigated. We observed that while LPA stimulated intracellular SIP synthesis, the inhibition of the sphingosine kinase blunted the inhibitory effects of LPA on steroid synthesis in LCp2 cells. In contrast to the LPA-induced effects, the inhibition of the sphingosine kinase had no effect on the attenuation of the steroidogenesis induced by the exogenous SIP. Because the inhibition of steroidogenesis varied between 60% and 90% in a single individual experiment, as a result of the heterogeneity of the primary cell system, the concomitant treatment with both lysolipids resulted in complete inhibition. This indicates that SIP and LPA may signal in a complementary way, rather than operating through the same pathway. In fact, exogenous SIP, unlike LPA, inhibited progesterone synthesis in a cyclic AMP-dependent way in both LCp2 and MA10 cells. The inhibi-


tion of the cyclic AMP is of particular interest, because the recent literature provides data relating to the mechanistic aspects of sustained inhibition of the cyclic AMP signaling by SIP (51). These authors provide strong evidence that SIP induces membrane translocation of protein associated with myc (PAM), which is one of the most potent inhibitors of the adenyl cyclase (51). Although PAM is highly expressed in the ovary, it remains to be determined in future studies whether this mechanism also takes place in steroidogenic cells. Although we could not determine the effect of LPA on the amounts of either sphingosine or *N*-acylated sphingosine, the possible contribution of other pathways to the intracellular SIP production should also be considered (52). Future studies should clarify whether sphingosine kinase activity is in fact regulated by LPA stimulation and whether this activity is correlated with the activation of PAM.

The temporally occurring attenuation of progesterone production in luteal cells requires further evaluation, because LPA did not inhibit steroid synthesis in any other ovarian cell type. In fact, LPA weakly stimulated progesterone production in theca cells, which express only LPA<sub>1</sub> receptor (25, 53).

The rate-limiting step of steroid synthesis is the transfer of cholesterol from the outer to the inner mitochondrial membrane, a process regulated functionally by the StAR protein (22, 23). We could not observe any effect of the lysolipid on in vitro pregnenolone synthesis in isolated mitochondria using membrane-permeable cholesterol substrates and could observe no effect on the expression of the StAR protein in mitochondria fractions. A functional effect on the StAR protein function cannot be completely excluded. Future research will, hopefully, throw more light upon these issues. Before the mitochondrial cholesterol side-chain cleavage enzyme initiates pregnenolone biosynthesis, several premitochondrial steps can accelerate the steroid synthesis in response to tropin stimulation (i.e., LH). These steps include the intracellular cholesterol transport, a pathway that provides the steroid precursor from intracellular stores, and the hydrolysis of the cholesteryl ester. Several aspects of the mechanisms regulating the in-



**Fig. 10.** Ovarian luteal (LC) and theca cells (Tk), but not MA10 cells express endogenous LPA<sub>1</sub> receptor protein. Membrane fractions from various luteal cell preparations (LCp1, LCp2, LCp3), theca, MA10, and HL-60 cells (negative control) were separated on SDS-PAGE, blotted to PVDF membranes, and incubated with a polyclonal antibody recognizing LPA<sub>1</sub> receptor as described in Materials and Methods. Chemiluminescence analysis was performed as detailed in Materials and Methods. Lower control bands show  $\beta$ -actin staining in rehybridized blot.

tracellular transport of cholesterol in steroidogenic tissues have already been elucidated and point to the role of microtubuli and the intermediate filaments (21). Signals regulating luteal cell migration and cell shape changes associated with the maturation and function of the corpus luteum are poorly understood. This transient reproductive organ is formed from mature ovarian follicles during a complex process known as follicular luteal transition (luteinization), comprising follicular cell differentiation associated with inflammatory and wound-healing-like events (54). Initiated by the hormonal stimulus (luteotropin LH), luteinization results in differentiation of the follicular granulosa and theca cells and formation of a new cell type, the luteal cell. The main function of luteal cells is to provide the steroid hormone progesterone, which initiates uterine glandularization and serves as a negative signal to the hypothalamus, suppressing further follicular development (55). Unless pregnancy interrupts the transient life span of the corpus luteum, steroid production ceases by the end of the estrous cycle (20). Although the gonadotropin LH is an important regulator of ovarian steroid production, there is abundant evidence that the effect of LH can be moderated by other hormones or by local factors (54, 55) produced within the ovary, such as LPA. The differential endogenous expression of LPA<sub>2</sub> and the constant presence of LPA<sub>1</sub> receptor may indicate multiple functions for LPA within the ovary. 

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## REFERENCES

- Ishii, J., N. Fukushima, X. Ye, and J. Chun. 2004. Lysophospholipid receptors: signaling and biology. *Annu. Rev. Biochem.* **73**: 321–354.
- Luquain, C., V. A. Sciorra, and A. J. Morris. 2003. Lysophosphatidic acid signaling: how a small lipid does big things. *Trends Biochem. Sci.* **28**: 377–383.
- Tokumura, A. 2002. Physiological and pathophysiological roles of lysophosphatidic acids produced by secretory lysophospholipase D in body fluids. *Biochim. Biophys. Acta.* **1582**: 18–25.
- Tokumura, A., Y. Kanaya, M. Miyake, S. Yamano, M. Irahara, and K. Fukuzawa. 2002. Increased production of bioactive lysophosphatidic acid by serum lysophospholipase D in human pregnancy. *Biol. Reprod.* **67**: 1386–1392.
- Mills, G. B., and W. H. Moolenaar. 2003. The emerging role of lysophosphatidic acid in cancer. *Nat. Rev. Cancer.* **3**: 582–591.
- Fukushima, N., I. Ishii, J. J. Contos, J. A. Weiner, and J. Chun. 2001. Lysophospholipid receptors. *Annu. Rev. Pharmacol. Toxicol.* **41**: 507–534.
- Contos, J. J., I. Ishii, and J. Chun. 2000. Lysophosphatidic acid receptors. *Mol. Pharmacol.* **58**: 1188–1196.
- Contos, J. J., I. Ishii, N. Fukushima, M. A. Kingsbury, X. Ye, S. Kawamura, J. H. Brown, and J. Chun. 2002. Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). *Mol. Cell. Biol.* **22**: 6921–6929.
- Contos, J. J., N. Fukushima, J. A. Weiner, D. Kaushal, and J. Chun. 2000. Requirement for the lpa1 lysophosphatidic acid receptor gene in normal suckling behaviour. *Proc. Natl. Acad. Sci. USA.* **97**: 13384–13389.
- Kingsbury, M. A., S. K. Rehen, J. J. Contos, C. M. Higgins, and J. Chun. 2003. Non-proliferative effects of lysophosphatidic acid enhance cortical growth and folding. *Nat. Neurosci.* **6**: 1292–1299.
- Ye, X., I. Ishii, M. A. Kingsbury, and J. Chun. 2002. Lysophosphatidic acid as a novel cell survival/apoptotic factor. *Biochim. Biophys. Acta.* **1585**: 108–113.
- Ishii, I., J. J. Contos, N. Fukushima, and J. Chun. 2000. Functional comparisons of the lysophosphatidic acid receptors, LP(A1)/VZG-1/EDG-2, LP(A2)/EDG-4, and LP(A3)/EDG-7 in neuronal cell lines using a retrovirus expression system. *Mol. Pharmacol.* **58**: 895–902.
- Huang, M. C., M. Graeler, G. Shankar, J. Spencer, and E. J. Goetzl. 2002. Lysophospholipid mediators of immunity and neoplasia. *Biochim. Biophys. Acta.* **23**: 161–167.
- Fang, X., S. Yu, R. C. Bast, S. Liu, H. J. Xu, S. X. Hu, R. LaPushin, F. X. Claret, B. B. Aggarwal, Y. Lu, and G. B. Mills. 2004. Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. *J. Biol. Chem.* **279**: 9653–9661.
- Fang, X., M. Schummer, M. Mao, S. Yu, F. H. Tabassam, R. Swaby, Y. Hasegawa, J. L. Tanyi, R. LaPushin, A. Eder, et al. 2002. Lysophosphatidic acid is a bioactive mediator in ovarian cancer. *Biochim. Biophys. Acta.* **1582**: 257–264.
- Tokumura, A., M. Miyake, Y. Nishioka, S. Yamano, T. Aono, and K. Fukuzawa. 1999. Production of lysophosphatidic acids by lysophospholipase D in human follicular fluids of in vitro fertilization patients. *Biol. Reprod.* **61**: 195–199.
- Hinokio, K., S. Yamano, K. Nakagawa, M. Irahara, M. Kamada, A. Tokumura, and T. Aono. 2002. Lysophosphatidic acid stimulates nuclear and cytoplasmic maturation of golden hamster immature oocytes in vitro via cumulus cells. *Life Sci.* **70**: 759–767.
- Budnik, L. T., and A. K. Mukhopadhyay. 2002. Lysophosphatidic acid and its role in reproduction. *Biol. Reprod.* **66**: 859–865.
- Budnik, L. T., and A. K. Mukhopadhyay. 2001. Lysophosphatidic acid antagonizes the morphoregulatory effects of the luteinizing hormone on luteal cells: possible role of small Rho-G-proteins. *Biol. Reprod.* **65**: 180–187.
- Niswender, G. D. 2002. Molecular control of luteal secretion of progesterone. *Reproduction.* **123**: 333–339.
- Hall, P. F., and G. Almahbobi. 1997. Roles of microfilaments and intermediate filaments in adrenal steroidogenesis. *Microsc. Res. Tech.* **36**: 463–479.
- Stocco, D. M. 2001. Tracking the role of a star in the sky of the new millennium. *Mol. Endocrinol.* **15**: 1245–1254.
- Stocco, D. M., and B. J. Clark. 1996. Regulation of the acute production of steroids in steroidogenic cells. *Endocr. Rev.* **17**: 221–244.
- Ireland, J. J., R. L. Murphee, and P. B. Coulson. 1980. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *J. Dairy Sci.* **63**: 155–160.
- Budnik, L. T., B. Brunswig-Spickenheier, and A. K. Mukhopadhyay. 2003. Lysophosphatidic acid signals through mitogen-activated protein kinase-extracellular signal regulated kinase in ovarian theca cells expressing the LPA1/edg2-receptor: involvement of a nonclassical pathway? *Mol. Endocrinol.* **17**: 1593–1606.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.
- Budnik, L. T., D. Jähner, and A. K. Mukhopadhyay. 1999. Inhibitory effects of TNF alpha on mouse tumor Leydig cells: possible role of ceramide in the mechanism of action. *Mol. Cell. Endocrinol.* **150**: 39–46.
- Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**: 911–917.



29. Freeman, D. A., and M. Ascoli. 1982. Studies on the source of cholesterol used for steroid biosynthesis in cultured Leydig tumor cells. *J. Biol. Chem.* **257**: 14231–14238.
30. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156–159.
31. Friedman, A., S. Weiss, N. Levy, and R. Meidan. 2000. Role of tumor necrosis factor alpha and its type I receptor in luteal regression: induction of programmed cell death in bovine corpus luteum-derived endothelial cells. *Biol. Reprod.* **63**: 1905–1912.
32. Tsai, S. J., M. C. Wiltbank, and K. J. Bodensteiner. 1996. Distinct mechanisms regulate induction of messenger ribonucleic acid for prostaglandin (PG) G/H synthase-2, PGE (EP3) receptor, and PGF2 alpha receptor in bovine preovulatory follicles. *Endocrinology*. **137**: 3348–3355.
33. Jones, M. J., and A. W. Murray. 1995. Evidence that ceramide selectively inhibits protein kinase C-alpha translocation and modulates bradykinin activation of phospholipase D. *J. Biol. Chem.* **270**: 5002–5013.
34. Rani, C. S., F. Wang, E. Fuior, A. Berger, J. Wu, T. W. Sturgill, D. Beitner-Johnson, D. LeRoith, L. Varticovski, and S. Spiegel. 1997. Divergence in signal transduction pathways of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors. *J. Biol. Chem.* **272**: 10777–10783.
35. Wang, F., C. D. Nobes, A. Hall, and S. Spiegel. 1997. Sphingosine 1-phosphate stimulates rho-mediated tyrosine phosphorylation of focal adhesion kinase and paxillin in Swiss 3T3 fibroblasts. *Biochem. J.* **324**: 481–488.
36. Budnik, L. T., and A. K. Mukhopadhyay. 1997. Functional lysophosphatidic acid receptor in bovine luteal cells. *FEBS Lett.* **419**: 4–8.
37. Goetzl, E. J., H. Dolezalova, Y. Kong, Y. L. Hu, R. B. Jaffe, K. R. Kalli, and C. A. Conover. 1999. Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. *Cancer Res.* **59**: 5370–5375.
38. An, S., T. Bleu, O. G. Hallmark, and E. J. Goetzl. 1998. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J. Biol. Chem.* **273**: 7906–7909.
39. Young, K. W., M. D. Bootman, D. R. Channing, P. Lipp, P. R. Maycox, J. Meakin, R. A. Challiss, and S. R. Nahorski. 2000. Lysophosphatidic acid-induced Ca<sup>2+</sup> mobilization requires intracellular sphingosine 1-phosphate production. Potential involvement of endogenous EDG-4 receptors. *J. Biol. Chem.* **275**: 38532–38539.
40. Simpson, P. B., I. R. Villullas, I. Schurov, J. Kerby, R. Millard, C. Haldon, M. S. Beer, and G. McAllister. 2002. Native and recombinant human Edg4 receptor-mediated Ca(2+) signalling. *Assay Drug Dev. Technol.* **1**: 31–40.
41. Shida, D., T. Watanabe, J. Aoki, K. Hama, J. Kitayama, H. Sonoda, Y. Kishi, H. Yamaguchi, S. Sasaki, A. Sako, et al. 2004. Aberrant expression of lysophosphatidic acid (LPA) receptors in human colorectal cancer. *Lab. Invest.* **84**: 1352–1362.
42. Huang, M. C., M. Graeler, G. Shankar, J. Spencer, and E. J. Goetzl. 2002. Lysophospholipid mediators of immunity and neoplasia. *Biochim. Biophys. Acta.* **1582**: 161–167.
43. Furui, T., R. LaPushin, M. Mao, H. Khan, S. R. Watt, M. A. Watt, Y. Lu, X. Fang, S. Tsutsui, Z. H. Siddik, et al. 1999. Overexpression of edg-2/vzg-1 induces apoptosis and anoikis in ovarian cancer cells in a lysophosphatidic acid-independent manner. *Clin. Cancer Res.* **5**: 308–318.
44. Huang, M. C., H. Y. Lee, C. C. Yeh, Y. Kong, C. J. Zaloudek, and E. J. Goetzl. 2004. Induction of protein growth factor systems in the ovaries of transgenic mice overexpressing human type 2 lysophosphatidic acid G protein-coupled receptor (LPA2). *Oncogene*. **2**: 122–129.
45. Fang, X., S. Yu, R. C. Bast, S. Liu, H. J. Xu, S. X. Hu, R. LaPushin, F. X. Claret, B. B. Aggarwal, Y. Lu, et al. 2004. Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. *J. Biol. Chem.* **279**: 9653–9661.
46. Xu, J., Y. J. Lai, W. C. Lin, and F. T. Lin. 2004. TRIP6 enhances lysophosphatidic acid-induced cell migration by interacting with the lysophosphatidic acid 2 receptor. *J. Biol. Chem.* **279**: 10459–10468.
47. Spiegel, S., and S. Milstien. 2003. Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. *Biochem. Soc. Trans.* **31**: 1216–1219.
48. Spiegel, S., and S. Milstien. 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat. Rev. Mol. Cell Biol.* **4**: 397–407.
49. Barber, S. C., H. Mellor, A. Gampel, and N. J. Scolding. 2004. S1P and LPA trigger Schwann cell actin changes and migration. *Eur. J. Neurosci.* **19**: 3142–3150.
50. Karliner, J. S., N. Honbo, K. Summers, M. O. Gray, and E. J. Goetzl. 2001. The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. *J. Mol. Cell. Cardiol.* **33**: 1713–1717.
51. Pierre, S. C., J. Hausler, K. Birod, G. Geisslinger, and K. Scholich. 2004. PAM mediates sustained inhibition of cAMP signaling by sphingosine-1-phosphate. *EMBO J.* **23**: 3031–3040.
52. Le Stunff, H., S. Milstien, and S. Spiegel. 2004. Generation and metabolism of bioactive sphingosine-1-phosphate. *J. Cell. Biochem.* **92**: 882–899.
53. Budnik, L. T., and A. K. Mukhopadhyay. 2002. Lysophosphatidic acid-induced nuclear localization of protein kinase C delta in bovine theca cells stimulated with luteinizing hormone. *Biol. Reprod.* **67**: 935–944.
54. Richards, J. S. 2001. Perspective: the ovarian follicle—a perspective in 2001. *Endocrinology*. **142**: 2184–2193.
55. Davis, J. S., and B. R. Rueda. 2002. The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. *Front. Biosci.* **7**: 1949–1978.