REVIEW

Lysophosphatidic acid LPA_{1-3} receptors: signaling, regulation and *in silico* analysis of their putative phosphorylation sites

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Lysophosphatidic acid (LPA) is a glycerophospholipid with a plethora of actions in the normal function of our organism as well as in the pathogenesis of many diseases. These actions are mainly exerted through a family of G protein-coupled receptors, which currently comprise six members; other receptors might participate in LPA actions including a nuclear receptor. In this work, we mainly focus on three of these receptors, i. e., LPA_{1-3}; those that were initially discovered which, have been more extensively studied and that are phylogenetically related among themselves, as well as with receptors for other bioactive phospholipids, such as those for spingosine 1-phosphate. The characteristics of these receptors, their patterns of tissue expression, and some of the actions in which they are involved are presented. Regulation of receptor function, including desensitization, internalization and phosphorylation has only been studied for the LPA_1 subtype. However, *in silico* analysis of potential phosphorylation sites indicate that all of these three receptors are putatively regulated by agonist activation and heterologous stimuli. We think LPA_{1-3} receptor regulation constitutes a niche of investigation that is potentially of great importance considering the physiological and pathophysiological actions in which they are involved.

Keywords: Lysophosphatidic acid; LPA; Lysophosphatidic acid receptors; receptor phosphorylation

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Introduction

Lysophosphatidic acid (LPA) is a not very abundant metabolite, which participates in the biosynthesis, catabolism and transport of phospholipids in the organism, and in the continuous turnover of membranous organelles, such as the plasma membrane, endoplasmic reticula or Golgi apparatus. Interestingly, LPA also plays another very important role as a mediator in cell communication in autocrine, paracrine and endocrine ways. Thus, LPA and other lipids are denominated "bioactive", meaning that they are extracellular messengers or local hormones.

The ability of LPA to increase smooth muscle contraction, blood pressure and platelet aggregation was discovered in the early 1980's [1]. It took almost ten years to frame the concept that LPA is an extracellular signal, contribution made in the early 1990's, by Wouter H.

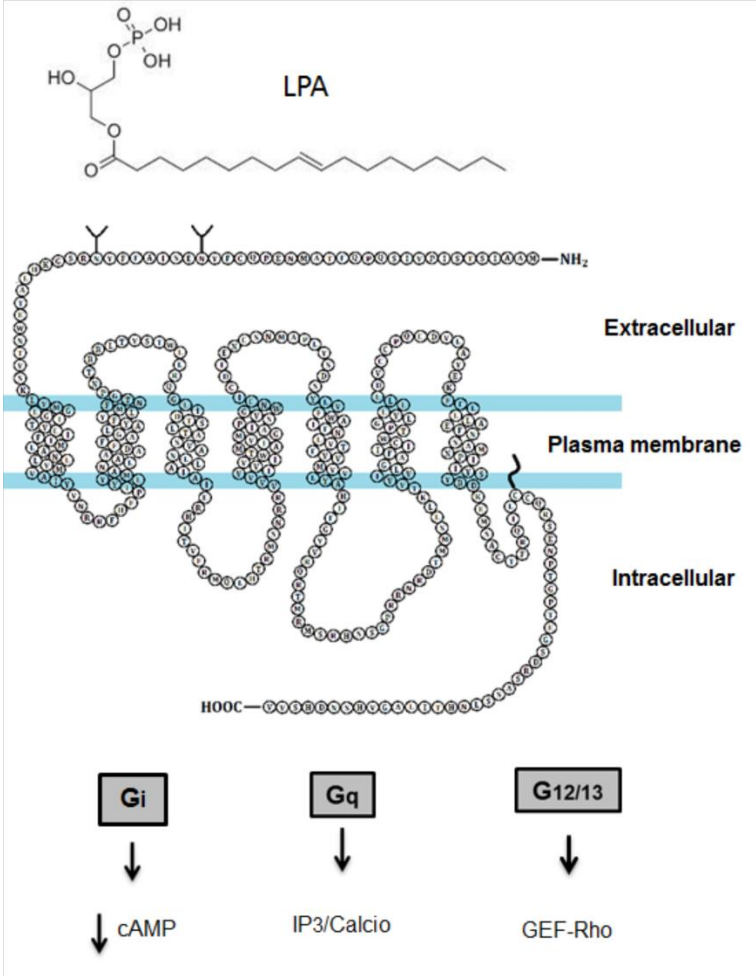

Fig 1. Model for the cellular action of lysophosphatidic acid through LPA$_{1-3}$ receptors. In the upper panel the molecular structure of a lysophosphatidic acid is presented. Middle and lower panels show the general structure of a LPA receptor (exemplified by LPA$_1$) and its interaction with different G proteins.

Moolenaar and his group [2, 3]. A few years later, the first receptor for this lipid was identified as the product of the ventricular zone gene-1 (vgz-1) by Chun and coworkers [4]. LPA is now recognized as a potent local mediator that exerts a plethora of actions regulating the normal function of our organism and also participating in the pathogenesis of different diseases. The physiological actions of LPA are enormously diverse and include: regulation of cardiac and smooth muscle cell contractility [5, 6], intestinal epithelial homeostasis [7], bone reabsorption [8, 9]. LPA also appears to be involved in fibrosis [10-12], lymphocyte homing and inflammation [13], cardiac hypertrophy [14], and in different types of cancer [15-21], and other morbid entities. At the cellular level, LPA modulates cytoskeletal organization [22], cell shape [23-26], proliferation [27], chemotaxis [28], cell migration and invasion[16], protection against or promotion of apoptosis (depending on cell type) [29-31], among other key processes. The amount of information on the actions of LPA is very abundant and continues growing at a very fast rate. However, there are important areas in which our knowledge is clearly insufficient. This paper is mainly focused on what is known and unknown about three LPA receptors, their signaling and regulation; *in silico* analysis of potential phosphorylation sites in these receptors is also presented. Many review papers are cited and readers are also directed to two special issues of Biochimica et Biophysica Acta [32, 33] and to the database and publications of the International Union of Basic and Clinical Pharmacology [34-38], which cover many aspects of LPA actions and receptors.

LPA represents an heterogeneous group of substances containing a free fatty acid joined through an ester bond to positions 1 or 2 of glycerol 3-phosphospate; the length of the fatty acid chain and its degree of saturation vary (Fig. 1). LPA can be generated both inside cells and

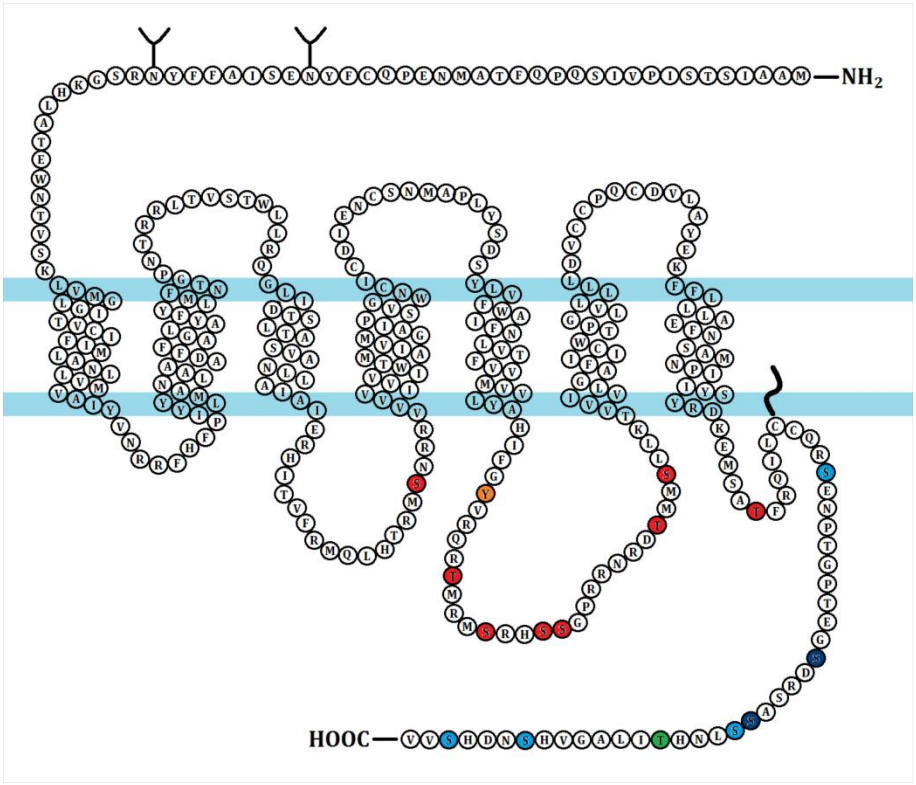

Fig 2. Schematic representation of the human LPA_1 receptor. Potential phosphorylation sites are indicated in colors with the kinase involved as follows: PKC, red; GRK, light blue; PKC and GRK, dark blue; PKA, yellow; Akt/PKB, green, and Tyrosine kinases, orange. For isozymes putatively involved and sites potentially targeted by several kinases, please see Table I. "Y" indicates potential glycosylation sites; "Z", potential palmitoylation sites; blue lines indicate plasma membrane region.

extracellularly, via the sequential action of phospholipases A_1/A_2 and D [39-41] and is degraded by several types of lipid phosphatases [41, 42]. LPA is present in tissues, in normal fluids such as blood, urine, saliva or cerebrospinal fluid, and also in pathological liquids, such as ascites [41, 43].

LPA can modulate transcription through nuclear receptors, such as the ligand-activated peroxisome proliferator-activated receptor γ (PPARγ) [44], an aspect that will not be addressed here. However, many of the major LPA actions are mediated through seven-transmembrane domains/ G protein-coupled receptors (GPCRs). Currently six GPCRs constitute the LPA receptor family [37], and are denominated LPA_{1-6} [34-36]. The orphan receptor GPR87, was recently shown to be activated by this lipid and was suggested as a new LPA receptor, i.e., LPA_7 [45]. All of these receptors interact during signaling with one or more G proteins (G_q, $G_{12/13}$, G_i or G_s). Three of these, LPA_{1-3}, were the first to be identified, are related phylogenetically among themselves, sharing ≈ 50% amino acid sequence identity[34-36, 40, 41], and also with other phospholipid receptors; they are the subject of this work (Figs. 1-4). The remaining LPA receptors, LPA_{4-7}, are relatively distant phylogenetically from the others but are related with the purinergic receptor family [34-36, 40, 41, 45]. In the Figures we have indicated with colors the sites that are possible targets of different protein kinases. Preference was given to sites affected by PKC, GRK or both, due to the fact that they are known regulators of LPA_1 [46-53]; when multiple kinases target a site, readers are referred to Tables I-III.

Despite the advances achieved in the field during the last 10 years, considerable gaps continue to persist in our knowledge. One of these is the lack of precise association of particular receptors with specific actions. Several reasons might explain this. An obvious one is that there is a great overlapping among the functions of these receptors [40]. This appears to be a general situation for many receptors and is likely the result of pressure selection during evolution, helping to assure species persistence. Lack of pharmacological tools has also contributed to the paucity of progress. Despite efforts made by many groups working in medicinal chemistry, the availability of potent and selective agonists and antagonists for these receptors is scarce, essentially limited to a few ligands with marginal

Table 1. Predicted human LPA_1 receptor phosphorylation sites.

Kinases	Number	S/T/Y	Sequence	Localization
PKA, PKC α, δ, η, CaMK 1	160	S	MQLHTRM**S**NRRVVVV	Intracellular loop 2
PDGFR, TYK 2, JAK, Abl	231	Y	LYAHIFG**Y**VRQRTMR	Intracellular loop 3
PKA, PKC α, β, δ, CaMK 1	236	T	FGYVRQR**T**MRMSRHS	Intracellular loop 3
PKA, PKC α	240	S	RQRTMRM**S**RHSSGPR	Intracellular loop 3
PKA, PKC α, CaMK 2	243	S	TMRMSRHS**S**GPRRNR	Intracellular loop 3
PKA, PKC α, δ, η, CAMK 4, AMPK	244	S	MRMSRHS**S**GPRRNRD	Intracellular loop 3
PKA, PKC α, CaMK 2	252	T	GPRRNRD**T**MMSLLKT	Intracellular loop 3
PKC ζ	255	S	RNRDTMM**S**LLKTVVI	Intracellular loop 3
PKB, PKC β, δ, θ, η, CaMK 1	321	T	RDKEMSA**T**FRQILCC	C-terminal
GRK 2	331	S	QILCCQR**S**ENPTGPT	C-terminal
PKC β, δ, θ, GRK 2, MAPK 13	341	S	PTGPTEG**S**DRSASSL	C-terminal
PKA, PKC δ, η, GRK3, CaMK 4, AMPK	346	S	EGSDRSA**S**SLNHTIL	C-terminal
GRK 3	347	S	GSDRSAS**S**LNHTILA	C-terminal
Akt/PKB	351	T	SASSLNH**T**ILAGVHS	C-terminal
GRK 2	358	S	TILAGVH**S**NDHSVV*	C-terminal
GRK 2	362	S	GVHSNDH**S**VV*****	C-terminal

The kinases that might target the site, amino acid number, amino acid, sequence and localization are indicated. Abbreviations used are: protein kinase C (PKC), G protein receptor kinase (GRK), protein kinase A (PKA), protein kinase B (Akt/PKB), mitogen-activated protein kinase (MAPK), calcium/calmodulin-dependent protein kinase (CaMK), AMP-dependent protein kinase (AMPK), PDGF receptor (PDGFR), tyrosine-protein kinase (TYK), Janus kinase (JAK), Abelson murine leukemia viral oncogene homolog (Abl).

selectivity [1, 40, 54-59]. Fortunately, information on the functions of these receptors has been gained through determination of messenger RNA expression in different tissues and the use of animals and cells in which the expression of one or more of these receptors has been blocked [60]. A very elegant proteomic analysis of LPA-induced protein phosphorylation has been reported, showing that this lysophospholipid induces marked changes in the phosphoproteome, with a change in ≈ 30% of the 6,292 phosphosites studied, and the time-course of the events suggested a protein kinase orchestration of signaling [61]. Interestingly, the authors concluded that the function of most LPA-regulated phosphorylation sites has not been investigated, which opens a new research avenues in the field [61].

Succinct information on the structural characteristics of these receptors, their signaling, tissue distribution and major actions is presented in the following paragraphs.

LPA_1 receptor

The human *LPAR1* gene is present in chromosome 9 site 9q31.3 and encodes a protein of 364 amino acids (molecular mass ≈ 41 kDa) [4, 37, 40, 62, 63] (Fig. 2). As indicated previously, this was the first receptor identified of this family [4], it has been considered prototypical for this receptor family and has been detected, in many human and mouse organs, such as brain, heart, intestine, ovaries, testis, prostate, thymus and pancreas, among others [40]. Signaling through this receptor appears to involve three G protein partners, $G\alpha_{i/o}$, which inhibit adenylyl cyclase, thus decreasing cyclic AMP levels; Gαq/11, which activate phospholipase C, increasing IP_3 and diacylglycerol formation and the concentration of intracellular calcium; or Gα12/13, which modulate the Rho pathway [40, 46, 51]. Through these pathways a variety of cellular responses are triggered; these include: cell proliferation, survival, cell shape changes and cytoskeletal rearrangements, migration, rearrangements in cell-cell contacts, among many others [40, 46, 51]. The LPA_1 third intracellular loop is critical for signaling [51] and a PDZ binding domain is present in the carboxyl terminus of LPA_1 receptors, which appears to participate in signaling [64, 65]. Not surprisingly, many of the known LPA effects are mediated through this subtype. These include actions that affect the central nervous system development and function, such as modulating embryonic Schwann cell migration, myelination and cell-to-axon segregation [66], astrocyte proliferation [67], formation of dendritic spine synapses [68] and also in neurite retraction [69], which can be involved in neuritogenesis. Consistent with these findings, LPA_1 receptor knockout mice exhibit synaptic dysfunction in the hyppocampus [70], reduced neurogenesis in the dentate gyrus [71] and defective cortical development [72]; these modifications are associated with altered suckling [73] and other behavioral abnormalities such as reduced wheel running and voluntary exercise [74], anxiety and spatial-memory impairments [75]. It has been suggested

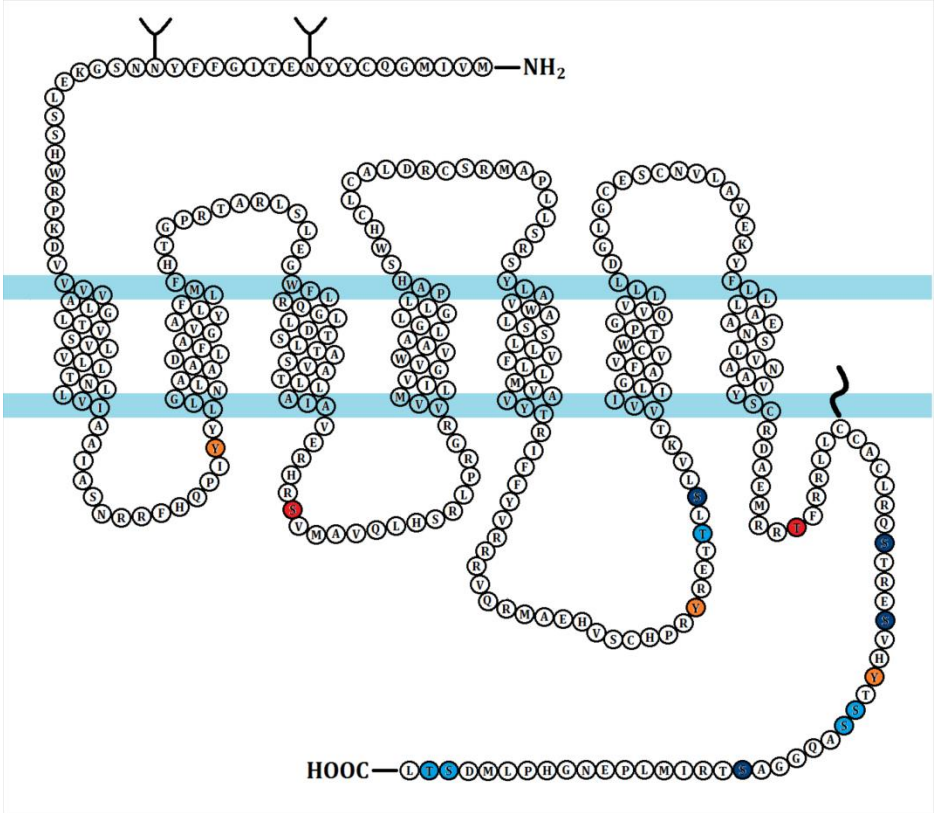

Fig 3. Schematic representation of the human LPA_2 receptor. Potential phosphorylation sites are indicated in colors with the kinase involved as follows: PKC, red; GRK, light blue; PKC and GRK, dark blue; PKA, yellow; and Tyrosine kinases, orange. For isozymes putatively involved and sites potentially targeted by several kinases, please see Table II. "Y" indicates potential glycosylation sites; "Z", potential palmitoylation sites; blue lines indicate plasma membrane region.

that the neurochemical changes observed in LPA_1-deficient mice could make them a possible model for psychiatric diseases, such as schizophrenia [76]. On the dark side, this receptor subtype participates in cerebral ischemia-induced neuropathic pain [77], hypoxia-induced stereotyped fetal brain disorganization [78], oxygen-induced retinal degeneration [79] and in the initiation of fetal hydrocephalus [80].

LPA_1 receptors play a role in osteoclast differentiation and bone reabsorption [8] and LPA_1 knockout mice show craniofacial dysmorfism [60] and abnormal bone development with decreased osseous tissue mass [9]. In zebrafish, this receptor subtypes appears to be essential for lymphatic vessel development [81], and in mice for angiogenesis [82]. LPA_1 appears to be involved in renal [10] and lung [11, 12] fibrosis, and in ovarian [18, 51], pancreatic [19, 20] and breast [15, 18] cancer, also increasing hepatocellular carcinoma cell-invasion [21]. LPA_1 receptor expression is increased while LPA_2 and LPA_3 are decreased, in ovarian cancer [51].

LPA_2 receptor

The LPA_2 receptor was discovered, taking advantage of its homology to LPA_1, through sequence search [83]. The human LPA_2 receptor gene, *LPAR2*, is located in chromosome 19 site 19p12 and encodes a protein of 351 amino acids (calculated molecular mass of ≈ 39 kDa) [37, 40] (Fig. 3). Its expression is restricted, as compared with LPA_1, but it has been detected in testis, leukocytes, prostate, spleen, intestine and pancreas [40]. This receptor also couples with $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ employing similar effector pathways to those of LPA_1. Additionally, it interacts with TRIP6, a focal adhesion molecule, and with other proteins, through a PDZ-binding domain present in its carboxyl terminus tail [40]. Interestingly, this receptor has been associated with cell survival and migration, and is considered a potential factor in cancer metastasis [16, 17]. In this regard it has been observed thatLPA_2 receptor activation promotes migration in gastric and ovarian cancer cells [16, 84] and in human colon cancer cells induces mitogenic signals [17], its presence is

Table 2. Predicted phosphorylation sites in the human LPA_2 receptor.

Kinases	Number	S/T/Y	Sequence	Localization
JAK 1, Blk	68	Y	RRFQPI**Y**YLLGNLA	Intracelular loop 1
PKA, PKC δ, PKC ζ	132	S	IAVERHR**S**VMAVQLH	Intracellular loop 2
VEGFR	232	Y	HVSCHPR**Y**RETTLSL	Intracelular loop 3
PKA, GRK 4	236	T	HPRYRET**T**LSLVKTV	Intracellular loop 3
PKC ζ, GRK 5	238	S	RYRETTL**S**LVKTVVI	Intracellular loop 3
PKA, PKB, PKC α, β, δ, θ	305	T	RDAEMRR**T**FRRLLCC	C-terminal
PKC α, β, θ, GRK 2	318	S	CCACLRQ**S**TRESVHY	C-terminal
PKA, PKC η, ζ, GRK 2	322	S	LQRSTRE**S**VHYTSSA	C-terminal
PDGFR, VEGFR, Yes	325	Y	STRESVH**Y**TSSAQGG	C-terminal
GRK 2, 3	327	S	RESVHYT**S**SAQGGAS	C-terminal
GRK 3	328	S	ESVHYTS**S**AQGGAST	C-terminal
PKC β, η, GRK 3	334	S	SSAQGGA**S**TRIMPLE	C-terminal
GRK 2	349	S	NGHPLMD**S**TL*****	C-terminal
GRK 2,	350	T	GHPLMDS**T**L*******	C-terminal

Indications as in Table I. Additional abbreviations: B lymphocyte kinase (Blk), VEGF receptor (VEGFR), c-yes protooncogene (Yes), Insulin receptor (InsR).

associated with ovarian cancer tumorigenicity and aggressiveness [51, 85] and mediates endometrial cancer invasion [86]. In a model of colitis-associated cancer, absence of LPA_2 receptors attenuated tumor formation [87].

LPA_3 receptor

The LPA_3 receptor was an orphan GPCR [88, 89]. Human *LPAR3* gene is located in chromosome locus 1p22.3-p31-1, it encodes a protein of 353 amino acids (estimated molecular mass of ≈ 40 kDa) [37, 40] (Fig. 4). Expression of this receptor subtype has been detected in brain, heart, pancreas, lung and in sex organs such as, testis, prostate, uterus (endometrium) and ovary [40, 51, 90]. LPA_3 receptors exert their actions through modulation of $G\alpha_{i/o}$ and $G\alpha_{q/11}$ function and consequently their activation inhibits adenylyl cyclase, decreasing cyclic AMP levels, it increases phospholipase C (phosphoinositidase) activity generating diacylglycerol, which increases protein kinase C (PKC) activity, and IP_3, which increases intracellular free calcium. Interestingly,this receptor subtype does not appears to couple with $G\alpha_{12/13}$, which modulates the Rho pathway [40]. LPA_3 knockout female mice show alterations in embryo implantation and small litter size [40, 91]; this seems to be mediated by phospholipase A_2 and cyclooxygenase activation, resulting in prostaglandin production and action in the endometrium [40, 90, 92]. There is little evidence for roles of LPA_3 receptors in nervous system functioning; however, recent evidence indicates that activation of this receptor subtype induces neurite branch formation, in neuronal cell lines [93]. Breast cancer aggressiveness has been associated with increased expression of LPA_3 and autotaxin [94].

Regulation of receptor function

GPCR function can be modulated by a very large number of processes with different mechanisms and time-frames. Changes in their steady state levels frequently involve modifications in their rates of synthesis and/ or degradation and represent long-term processes. However, changes in cell responsiveness to particular stimuli can be achieved rapidly within the range of minutes. Desensitization, defined operationally as diminished response to agonists, is a frequent adaptive response, that is induced by prolonged or repeated stimulation, but that can also be the result of brief receptor activation (homologous desensitization). Desensitization can also be induced rapidly by stimulation with agents unrelated with the receptor affected (heterologous desensitization). These desensitizations are frequently associated with covalent modification of receptors and/ or of other molecular entities involved in their signaling, and can also involve changes in the receptor's cellular localization (plasma membrane vs. intracellular vesicles) [95, 96]. It is currently accepted that receptor phosphorylation is an initial event in desensitization that subsequently leads to GPCR internalization [95, 97]. In addition, it is currently accepted that homologous desensitization-associated receptor phosphorylation is mainly catalyzed by G protein-coupled receptor kinases (GRKs) [98] whereas receptor phosphorylation caused by heterologous stimuli is catalyzed by second messenger-activated kinases, such as protein kinase A (PKA) [99, 100] and PKC [101-108], and also by other kinases of several different families, such as protein kinase B (Akt/ PKB) [109] , calcium/ calmodulin kinase 2 (CaMK2) [110] , MAP kinases [111], as well as by

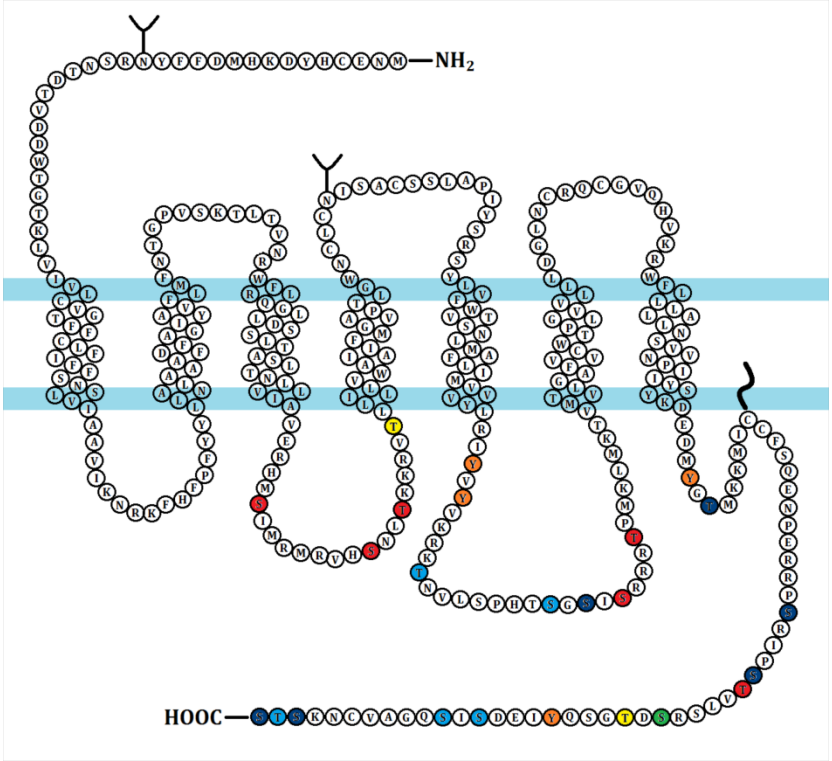

Fig 4. Schematic representation of the human LPA_3 receptor. Potential phosphorylation sites are indicated in colors with the kinase involved as follows: PKC, red; GRK, light blue; PKC and GRK, dark blue; PKA, yellow; Akt/PKB, green, and Tyrosine kinases, orange. For isozymes putatively involved and sites potentially targeted by several kinases, please see Table III. "Y" indicates potential glycosylation sites; "Z", potential palmitoylation sites; blue lines indicate plasma membrane region.

non-receptor and receptor tyrosine kinases [112-116]. However, it is noteworthy to mention that desensitization can also take place in the absence of receptor phosphorylation because receptor interaction with different proteins might constitute a steric hindrance for efficient G protein coupling [117]. It is important to consider that, during desensitization a particular response is diminished but the process frequently represents a switch in signaling, initiating other cellular events [116, 118-122]. Not all GPCR phosphorylations are associated to desensitization; for example, it has been observed that Akt/PKB-catalyzed phosphorylation of sphingosine 1-phosphate receptor 1 ($S1P_1$), at threonine 236, is required for its effect on endothelial migration, but not for other actions [123].

Current classic models for GPCR desensitization/ internalization suggest that receptor activation (homologous), induces a conformational change that is transmitted to G proteins [124], leading to GDP-GTP exchange and G protein subunit dissociation. This promotes GRK binding which facilitates receptor phosphorylation. These ATP-dependent phospho-transfer reactions take place mainly on serine and threonine (but can also include tyrosine) residues present in the third intracellular loop and carboxyl terminus. β-Arrestins bind to the phosphorylated receptor, initiating the internalization process and setting G protein-independent signaling into motion [99, 101-105]. In the case of heterologous desensitization, second messeger-activated and other kinases phosphorylate consensus sites in different unstimulated GPCRs. To date, it is far from clear to what extent such phosphorylations lead to β-arrestin binding and even less is known regarding G protein-independent signaling. What it is known, is that many GPCRs can be phosphorylated, desensitized, and internalized by both, agonist-stimulation and in a heterologous manner. The concepts of homologous and heterologous desensitizations are operationally correct and useful, but at the molecular level the events are more complex (see later).

In many cases where there is evidence of GPCR phosphorylation, the phosphorylation sites remain unknown. Site-directed mutagenesis and mass spectrometry of purified receptors are the major techniques employed. Very interesting data from Tobin

Table 3. Predicted phosphorylation sites in the human LPA_3 receptor.

Kinases	Number	S/T/Y	Sequence	Localization
PKA, PKC α, δ, ζ	130	S	IAVERHM**S**IMRMRVH	Intracelular loop 2
PKA, PKC α, ζ	138	S	IMRMRVH**S**NLTKKRV	Intracelular loop 2
PKC α	141	T	MRVHSNL**T**KKRVTLL	Intracelular loop 2
PKA	146	T	NLTKKRV**T**LLILLVW	Intracelular loop 2
Pyk 2	210	Y	VVVYLRI**Y**VYVKRKT	Intracellular loop 3
Pyk 2, JAK	212	Y	VYLRIYV**Y**VKRKTNV	Intracellular loop 3
PKA, GRK 5	217	T	YYYVKRK**T**NVLSPHT	Intracellular loop 3
GRK 4	225	S	NVLSPHT**S**GSISRRR	Intracellular loop 3
PKB, PKC α, ζ, GRK 4	227	S	LSPHTSG**S**ISRRRTP	Intracellular loop 3
PKC α, β, γ, δ, η	229	S	PHTSGSI**S**RRRTPMK	Intracellular loop 3
PKA, PKC α	233	T	GSISRRR**T**PMKLMKT	Intracellular loop 3
Fgr, Fyn, Yes, Lyn, Lck, JAK 2, VEGFR	301	Y	SYKDEDM**Y**GTMKKMI	C-terminal
PKC θ, GRK 4, 5	303	T	KDEDMYG**T**MKKMICC	C-terminal
PKA, PKC α, ζ, GRK 3	321	S	ENPERRP**S**RIPSTVL	C-terminal
PKA, PKC α, ε, GRK 3, 5	325	S	RRPSRIP**S**TVLSRSD	C-terminal
PKC γ	326	T	RPSRIPS**T**VLSRSDT	C-terminal
Akt/PKB	331	S	PSTVLSR**S**DTGSQYI	C-terminal
PKA	333	T	TVLSRSD**T**GSQYIED	C-terminal
Fyn, Yes, VEGFR, PDGFR, InsR	337	Y	RSDTGSQ**Y**IEDSISQ	C-terminal
GRK 2	341	S	GSQYIED**S**ISQGAVC	C-terminal
GRK 2	343	S	QYIEDSI**S**QGAVCNK	C-terminal
PKC γ, GRK 2, 3	351	S	QGAVCNK**S**TS******	C-terminal
GRK 2	352	T	GAVCNKS**T**S*******	C-terminal
PKC γ, δ, ζ, GRK 1, 2, 4, 5	353	S	AVCNKST**S********	C-terminal

Indications as in Tables I and II. Additional abbreviations: VEGF receptor (VEGFR), proline-rich tyrosine kinase (Pyk), feline sarcoma viral oncogene homolog (Fgr), tyrosine protein kinases Fyn (Fyn), Lyn, (Lyn) and Lck (Lck).

and coworkers have shown that the M_3 muscarinic acetyl choline receptor phosphopeptide maps differ in cell context-dependent fashion [110, 125-127]. Using different experimental tools the authors were also able to define that the muscarinic receptor phosphorylation varied in the same cell depending on the stimulus [125]. The possibility that different phosphorylation "bar codes" might explain varied functional responses, has been advanced [110, 125-127].

Regulation of LPA_{1-3} receptor function

To the best of our knowledge, the only LPA receptor known to be subjected to phosphorylation is LPA_1 [46-49]. Agonist stimulation and activation of PKC induce LPA_1 receptor phosphorylation [46-50]. Conventional PKC isozymes α and β, seem to play major roles in LPA_1 phosphorylation and desensitization induced by phorbol esters [47, 50]. This family of protein kinases also participates in 17 β-estradiol-induced LPA_1 desensitization and phosphorylation, in a signaling cascade that involves the estrogen receptor α and phosphoinositide 3-kinase (PI3K) activity [49]. Similarly, it has been observed that angiotensin II and EGF are also capable of inducing LPA_1 receptor phosphorylation and desensitization [47, 48]. Interestingly, LPA and angiotensin II induce plasma membrane HB-EGF shedding, which activates EGF receptors in autocrine/ paracrine fashion, and this contributes to LPA_1 phosphorylation and desensitization. Needless to say these findings indicate that homologous desensitization is not exclusively mediated through GRK. Similar obsevations have been made for the α_{1B}-adrenergic receptor [128-131]. Heterologous desensitization is also complex at the molecular level because there is an intense crosstalk between PKC and other signaling kinases (such as Src or the MAP kinases) and also GRKs [132-136]; therefore, it is possible that GRKs could participate in some "heterologous" desensitizations. The intense liason between the EGF receptors and LPA_1 also involves Akt/PKB; i.e., LPA induces Akt/PKB phosphorylation / activation (as reflected by threonine 308 phosphorylation) but this effect is mediated through the EGF receptor via HB-EGF membrane shedding [48]. This indicates that, on the one hand, some LPA-induced effects might involve EGF receptor stimulation but that, on the other hand, EGF activation leads to LPA_1 receptor desensitization.

Cell treatments with LPA or phorbol esters induce LPA_1 receptor internalization [51-53]. LPA-induced receptor internalization takes place via a β-arrestin, dynamin2- and Rab5-dependent pathway[51-53] whereas the phorbol ester-induced effect requieres clathrin and the AP-2 complex, but not β-arrestin [53]. Using a series of truncation mutants it was observed that a serine-rich domain (amino acids 341-347, SDRSASS) was required for agonist-induced internalization, while a dileucine motif (amino acids 352-353, IL) was required for the phorbol ester-induced effect [53]. These data indicate that the structural requirements for LPA- and phorbol ester-induced LPA_1 receptor

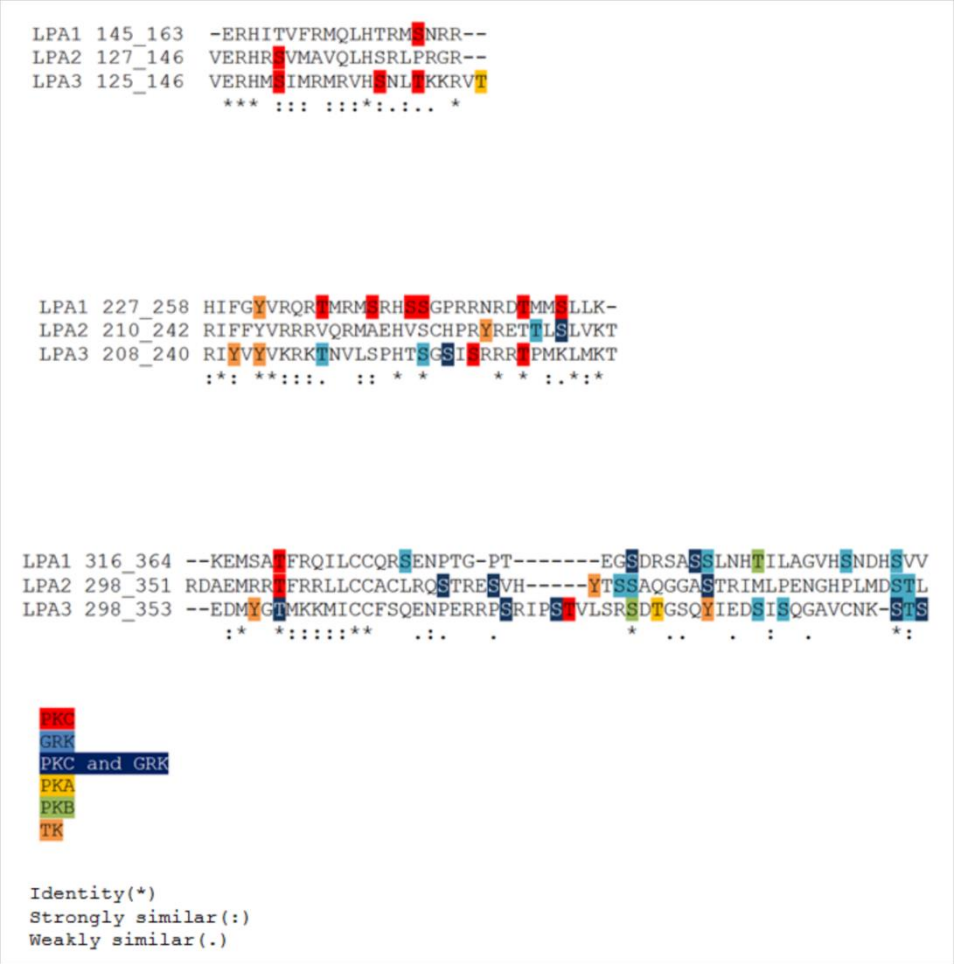

Fig 5. Alignment of intracellular loops 2 (upper), 3 (middle), and carboxyl temini (lower) of LPA_{1-3} receptors. Potential phosphorylation sites are indicated in colors with the kinase involved as follows: PKC, red; GRK, light blue; PKC and GRK, dark blue; PKA, yellow; Akt/PKB, green, and Tyrosine kinases, orange. For isozymes putatively involved and sites potentially targeted by several kinases, please see Tables I-III.

internalization are different [53]. The causal relationship among receptor phosphorylation , desensitization and internalization has not yet been firmly stablished. The small GTPase, Ral, and GRK2 regulate LPA_1 signaling [137]. Interestingly, the effect of GRK2 does not appear to requiere catalytic activity, because a dominant-negative mutant of this kinase also induces LPA_1 desensitization [137]. As previously mentioned LPA_1 contains a PDZ-binding domain in its carboxyl tail and it is through this domain that it interacts with PDZ-containing GIPC (GTPase activating protein [RGS19]-interacting protein) [64]. This binding promotes LPA_1 vesicular trafficking into early endosomes and attenuates signaling [64]. PDZ-binding domain-lacking LPA_1 receptors are constitutively active, stimulate cell proliferation and lead to colony formation under serum-free conditions [65].

Much less is known about LPA_2 receptor regulation. This receptor subtype also harbors a PDZ-binding domain in its carboxyl tail. Expression of these receptors increases GIPC basal activity, but the agonist, LPA, was unable to intensify such activity [137]. Interestingly LPA_2 (but not LPA_1) associates with a PDZ domain-containing scafoldding protein, i. e. the Na^+/H^+ exchanger regulatory factor 2; this association appears to increase receptor signaling through phospholipase C [138] and channel function [139]. Another PDZ-containg protein, MAGI-3, also interacts with LPA_2 and, in this case, exerts a negative modulation of signaling [140]. In other words, LPA_2 signalling is modulated, in an opposing manner, by two PDZ-containing proteins. Other proteins also interact with the LPA_2 carboxyl tail [141].

Information on LPA_3 regulation is limited to the modulation of its genetic expression, during the menstrual cycle through the action of progesterone and estrogen [92, 142, 143].

Analysis of putative LPA_{1-3} phosphorylation sites

Receptor sequences obtained from the UniProt database [144, 145] [LPA_1 (Q92633, LPAR1_HUMAN), LPA_2 (Q9HBW0, LPAR2_HUMAN), and LPA_3 (Q9UBY5, LPAR3_HUMAN)], were subjected to alignments and analysis of putative phosphorylation sites using the Group Based Prediction System (GPS algorithm 2.1 v) [146, 147] and the Swiss Institute of Bioinformatics, ExPASy-ClustalW [148, 149], proteomic programs. The GPS algorithm is based on the hierarchical structure of protein kinases and the partition of the phosphorylation sites known into several groups. A phosphorylation site is predicted if it possesses significant sequence similarity to known phosphorylation sites in at least one group. The GPS program indicates the predicted phosphorylated peptide, the kinases most likely able to catalyze the phosphorylation, and a numerical score for each protein kinase [146]. It is noteworthy that there is redundancy, i. e., many possible phosphorylation sites are putative targets of more than one protein kinase; this is indicated in Tables I-III, in which predicted phosphorylation sites for LPA_{1-3} receptors are presented. Sites were analyzed for the following protein kinases: PKC isoforms, PKA, GRKs, Akt/PKB, CaMK, AMP-dependent protein kinase (AMPK), mitogen-activated protein kinases (ERK/MAPK), and tyrosine kinases. The majority of the predicted phosphorylation sites of LPA_{1-3} receptors are located in their third intracellular loops and carboxyl termini, although some sites were also predicted in the first and second intracellular loops (Tables I-III). *In silico* analysis evidenced marked differences in predicted phosphorylation sites among the three receptors (Tables I-III, see also Fig. 5).

Phosphorylation predictions indicate that LPA_1 could be a target of six PKC isozymes α, β, δ, ζ, η and θ), two GRK isozymes (GRK 2 and 3 [some sites were possible targets of GRK 1(rhodopsin kinase) in the receptors analyzed but this was omitted due to the GRK 1limited tissue expression [98]]), PKA, and Akt/PKB (Table I and Figs. 2 and 5). Interestingly, most PKC target sites are within the third intracellular loop whereas most GRK-target sites are in the carboxyl terminus.

The abundance of putative phosphorylation sites in LPA_2 is only slightly less than that in LPA_1 and it is much higher in LPA_3 (Figs. 2-5 and Tables I-III). Potentially, LPA_2 could be phosphorylated by PKC α, β, δ, ζ, θ, and η, on sites in the second and third intracellular loops; similarly, GRK 2, 3, 4 and 5, could phosphorylate sites but these are present in the carboxyl terminus of this receptor. Some putative PKA phosphorylation sites are present in the third intracellular loop and carboxyl terminus of LPA_2 (Figs. 3 and 5, and Table II).

As already mentioned, the number of possible phosphorylation sites in LPA_3 is very high, i.e., 24 possible sites. Eight PKC isozymes (α, β, γ, δ, ε, ζ, θ and η) could potentially phosphorylate sites distributed in LPA_3 second and third intracellular loops, and also in the carboxyl terminus (Table III and Figs. 4 and 5). Putative phosphorylation sites for GRK2, 3, 4 and 5 isozymes are present mainly in the carboxyl terminus and possible targets of PKA and Akt/PKB also exist in LPA_3 (Table III and Figs. 4, and 5). Putative phosphorylation sites for receptor and non-receptor tyrosine kinases were detected in different domains of the three receptors (Figs. 2-5 and Tables I-III). It is remarkable that many putative PKC sites are in the third intracellular loops whereas the majority of GRK sites are in the carboxyl termini of these receptors. Putative tyrosine phosphorylation sites also exist, for the three receptors (Tables I-III and Figs. 2-5).

Alignment of LPA_{1-3} receptors analyzed with the Clustal-W program [148] showed that amino acid identity for the second intracellular loops was ≈ 23 %, for third intracellular loops ≈ 28%, and for the carboxyl termini, only 9% (Fig. 5).

Despite this low amino acid sequence identity, alignment showed that three potentially phosphorylatable residues are conserved in the carboxyl termini of the three receptor subtypes. These include the following: a) T321 (LPA_1), T305 (LPA_2) and T303 (LPA_3), targeted in common by PKC and individually other protein kinases; b) S341 (LPA_1), S328 (LPA_2) and S331(LPA_3), targeted by different protein kinases; and c) S362 (LPA_1), S349 (LPA_2) and S351(LPA_3), targeted in common by GRK 2 and the site in LPA_3 also by PKC. There are also some conserved sites in which the GPS programs predict phosphorylation of only two of these (indicated in the same order, non-predicted to be phosphorylation targets in brackets): a) Y231 (LPA_1), [Y214] (LPA_2) and Y212 (LPA_3); b) S244 (LPA_1), [S227] (LPA_2) and S225 (LPA_3); and c) T252 (LPA_1), [T235] (LPA_2) and T233 (LPA_3). A putative phosphorylation site was detected in which the targeted amino acid changed in one receptor: S347 (LPA_1), S334 (LPA_2) and Y337 (LPA_3). The putative phosphorylations sites present in only two of these receptors include: a) S132 (LPA_2) and S130 (LPA_3); b) S255 (LPA_1) and S238 (LPA_2); and T350 (LPA_2) and T352 (LPA_3).

It was surprising to observe the presence of many putative PKA phosphorylations sites in the three LPA receptors studied; many of these were targets of different PKC isozymes.

It is clear that the *in silico* analysis presents a complex panorama, with many possible phosphorylable sites and redundant action of different kinases. It offers, as well, the

possibility of studying the role(s) of specific sites in receptor regulation by means of different conditions and stimuli. Obviously, this requires factual experimental demonstration. The use of mutants (truncated or site-directed) and mass spectrometry appears to be required to address this. It should be borne in mind that these processes take place in four dimensions, where location (3D) and time play key roles. The redundancy observed might also suggests the possibility that a given kinase or isoform might act on the receptor's susceptible sites during an initial time-frame, and then other kinase(s) might participate. It is also important to consider that receptor phosphorylation and the resulting phosphorylation bar-codes are likely very dynamic, resulting from the coordinate action of kinases and phosphatases, possibly reaching different short-lived signaling states. One can envision the study of these processes *in cellulo* through the use of microscopy, fluorescent probes and FRET and BRET techniques [150-152]. We are convinced that although the amount of information on LPA receptors is already very abundant, there are areas, such as receptor regulation, that offer niches of opportunity to many researchers and that are, in our opinion, relevant for understanding cell physiology and pathophysiology and promising for the development of better treatments of human and animal diseases.

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