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N-Palmitoyl-serine and *N*-Palmitoyl-tyrosine Phosphoric Acids Are Selective Competitive Antagonists of the Lysophosphatidic Acid Receptors

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SUMMARY

Lysophosphatidic acid is the best characterized member of a lipid mediator family with growth factor-like activities that act through a class of G protein-coupled plasma membrane receptors. In *Xenopus laevis* oocytes, lysophosphatidate activates at least two pharmacologically distinct receptor subtypes distinguished by 1-acyl-*sn*-glycero-2,3-cyclic phosphate. Both of these naturally occurring ligands elicit oscillatory Cl^- currents in the oocyte through G protein-coupled activation of the phosphoinositide/Ca^{2+} second messenger system, which in turn leads to the opening of Ca^{2+}-activated Cl^- channels. We developed an improved chemical synthesis and purification procedure for two *N*-acylated amino acid phosphates. *N*-Palmitoyl-serine and *N*-palmitoyl-tyrosine phosphoric acids inhibited the lysophosphatidate-activated Cl^- currents with IC_{50} values of 5.4 ± 0.7 and 6.5 ± 1.5 nM at the high affinity site and 805 ± 97 and 172 ± 36 nM at the low affinity receptor site, respectively. In selective activation of the cyclic lysophosphatidate receptor, IC_{50} values of 330 ± 30 and 490 ± 40 nM were obtained, respectively. The D- and L-stereoisomers were equally effective when applied extracellularly. In contrast, they were ineffective when microinjected into the oocyte, indicating an extracellular site of inhibition. The inhibitors did not alter currents elicited by the different acetylcholine, serotonin, and glutamate receptors expressed heterologously in the oocyte. Pharmacological analysis of the results indicates that *N*-palmitoyl-serine and *N*-palmitoyl-tyrosine phosphoric acids are potent and specific competitive inhibitors of the lysophosphatidate receptors in the *X. laevis* oocyte.

LPA has emerged as a potent lipid mediator with growth factor-like biological activities (reviewed in Ref. 1). LPA bound to albumin is a normal constituent of vertebrate serum generated during blood clotting from activated platelets (2–4). Receptor-mediated responses to LPA have been detected in a variety of cell types, including fibroblasts (5), *Xenopus laevis* oocytes (6), PC12 pheochromocytoma cells (7–11), neuronal cells (12), brain homogenates (13), several different carcinoma cells (7, 14), myoblasts (15), and human platelets (16). Many properties of the LPA receptors and characteristics of their signaling were first derived from studies using oocytes. In the oocyte, extracellularly applied LPA elicits dose-dependent oscillatory Cl^- currents through a G protein-mediated activation of the phosphoinositide/Ca^{2+} second messenger system, whereas it is ineffective when applied intracellularly (3, 6, 17–20). The structure-activity analysis of the interaction between lipid phosphatidates and their specific receptors derived from experiments carried out in oocytes (3) has been confirmed in many types of normal and transformed mammalian cells (13, 16, 21, 22). Using cLPA, a naturally occurring cyclic phosphate-containing LPA analog, we recently demonstrated the simultaneous expression of at least two distinct receptors for the LPA-like lipid mediator family (20). These receptors were classified as type I, responding to LPA but not activated by cLPA, and type II, responding to both LPA and cLPA (20). The two receptors are coupled to partially different heterotrimeric G protein-linked signaling pathways. Although their application is accompanied by the activation of phosphoinositide turnover, cLPA elevates, whereas LPA decreases, cAMP levels (23). Consequently, LPA is mitogenic, whereas cLPA is antimitogenic in the same fibroblast cell line (23).

The lack of highly selective antagonists to the LPA receptors has been a major obstacle to research aimed at elucidating the physiological role of this phylogenetically highly con-

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ABBREVIATIONS: LPA, 1-acyl-2-lyso-*sn*-glycero-3-phosphate; 18:1-LPA, 1-oleoyl-2-lyso-*sn*-glycero-3-phosphate; cLPA, 1-*O*-[(9′*S*,10′*R*)-9′,10′-methanohexadecanoyl]-*sn*-glycero-2,3-cyclic phosphate; 16:0-GP, 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NP-Ser-PA, *N*-palmitoyl-L-serine phosphoric acid; NP-Tyr-PA, *N*-palmitoyl-L-tyrosine phosphoric acid; PAF, platelet activating factor.

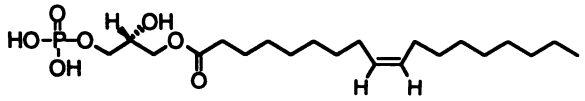
served group of lipid mediators. The pioneering steps in elucidation of the structural requirements for antagonists were performed by Sugiura *et al.* (16), who reported that *N*-palmitoyl-2-aminoethanol phosphoric acid induced aggregation in human platelets but that two other *N*-acylated compounds, NP-Ser-PA and NP-Tyr-PA, exhibited inhibitory effects on aggregation evoked by 16:0-GP, an alkyl analog of the acyl-LPA. They also demonstrated that these *N*-acylated compounds, as well as the alkyl-LPA, act on a receptor site that is distinct from the PAF receptor (16). In human platelets, PAF receptor antagonists were unable to block the agonist activity of 16:0-GP and *N*-palmitoyl-2-aminoethanol phosphoric acid, and NP-Ser-PA and NP-Tyr-PA did not interfere with the PAF-evoked platelet aggregation. Recently, we developed an improved stereospecific chemical synthesis of these compounds and reported their characterization with HPLC, fast atom bombardment mass spectrometry, and NMR spectroscopy (24). The availability of these *N*-acyl amino acid phosphates permitted the detailed pharmacological analysis of their mechanism of action in the *X. laevis* oocyte. The oocyte is ideal for studying the pharmacology of putative LPA receptor antagonists because 1) it is highly sensitive [the threshold concentration of 18:1-LPA is in the subnanomolar range (20)]; 2) the responses to the different ligands are easily quantified by measuring the oscillatory Cl^- currents with voltage-clamp recording (3, 6, 17–20); 3) oocytes do not express functional receptors to other lipid mediators, including PAF (3), sphingosine-1-phosphate (20), and lysosphingomyelin;[1] and 4) the expression of functional receptors for different families of modulators can be induced by microinjection of mRNA.

We report on the pharmacological characterization of the effects of NP-Ser-PA and NP-Tyr-PA on the LPA- and cLPA-elicited oscillatory Cl^- currents in *X. laevis* oocytes. NP-Ser-PA and NP-Tyr-PA both inhibited the currents in a reversible fashion by shifting the dose-response curves of 18:1-LPA to the right. They also inhibited the 18:1-LPA- or cLPA-elicited currents with IC_{50} values in the nanomolar range. The D- and L-stereoisomers were equally effective when applied extracellularly; however, they were ineffective when microinjected into the oocyte. The inhibitors did not alter currents elicited by the different acetylcholine, serotonin, and glutamate receptors expressed heterologously in the oocyte. These results indicate that NP-Ser-PA and NP-Tyr-PA are potent and specific competitive inhibitors of the lysophosphatidate receptors in the *X. laevis* oocyte.

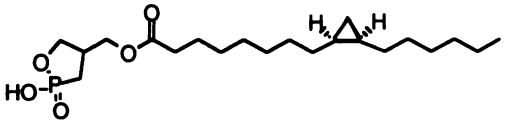
Experimental Procedures

Chemicals. The chemical structures and space-filling molecular models of the agonists and antagonists used in the present study are illustrated in Fig. 1 and 2, respectively. The space-filling molecular models were built using the SYBYL software package (Tripos Associates, St. Louis, MO) running on a Sun 4–260 workstation (Sun Microsystems, Sunnyvale, CA) and displayed on an Evans and Sutherland PS390 graphics display terminal.

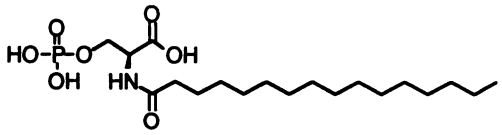
18:1-LPA and PAF were purchased from Avanti Polar Lipids (Birmingham, AL). Naturally occurring cLPA was synthesized as described previously (25). A new synthesizing strategy of NP-Ser-PA and NP-Tyr-PA was developed (24) that is based on the use of the benzyl ester of L-serine or L-tyrosine as the starting material. Briefly,



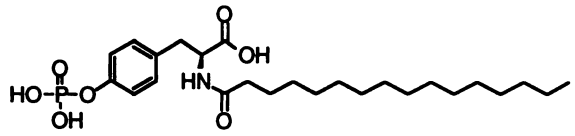
1-oleoyl-2-lyso-*sn*-glycero-3-phosphate, oleoyl-LPA



1-*O*-[(9'*S*,10'*R*)-9',10'-methanohexadecanoyl]-*sn*-glycero-2,3-cyclic phosphate, cLPA



N-palmitoyl-L-serine-phosphoric acid, *N*P-Ser-PA



N-palmitoyl-L-tyrosine-phosphoric acid, *N*P-Tyr-PA

Fig. 1. Chemical structures of lipid phosphatidates and their antagonists. *Oleoyl-LPA*, 18:1-LPA.

the first step was the *N*-acylation of the benzyl ester-protected amino acids using an activated palmitic acid; this was followed by the formation of the phosphate moiety by *O*-phosphitylation and oxidation. The final step involved the simultaneous debenzylation of both the phosphate and carboxylic acid esters. The procedure, which has the advantage of using standard protection/deprotection steps, converts the L-benzyl esters to NP-Ser-PA and NP-Tyr-PA with a 56% and 57% overall yield, respectively. The synthesis of the D-stereoisomers of the inhibitors was carried out the same way, using the D-benzyl esters of the amino acids at the start.

All other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless stated otherwise. The purity of the lipid phosphatidic acids was checked by high performance liquid chromatography on a Microsorb 5-μm silica column (Rainin, Woburn, MA) using an evaporative light-scattering detector (ELSD-IIA; Varex, Burtonsville, MD) and a two-pump solvent delivery system (Waters, Milford, MA). A straight-phase gradient solvent system described by Becart *et al.* (26) was used in the case of LPA and cLPA, and a modification of this method was used for final purification of NP-Ser-PA and NP-Tyr-PA (24). All of the compounds tested were of ≥95% purity based on high performance liquid chromatography.

Electrophysiological recording. Oocytes were obtained from adult *X. laevis* frogs (Xenopus I, Ann Arbor, MI) and prepared for experiments as described previously (6). Electrophysiological recording was carried out between 1 and 5 days after defolliculation of the oocytes with collagenase. Oocytes were voltage-clamped at −60 mV with a standard two-electrode voltage-clamp amplifier (GeneClamp 500; Axon Instruments, Burlingame, CA) during superfusion with frog Na^+/Ringer's solution (27) containing 5 mM HEPES, pH 7.0, 120 mM NaCl, 2 mM KCl, and 1.8 mM $CaCl_2$. Membrane currents were monitored with an NIC-310 digital oscilloscope (Nicolet, Madison, WI) and analyzed by the RICO software package, version 1.01 (Irvine CA).

[1] G. Tigyi, unpublished observations.

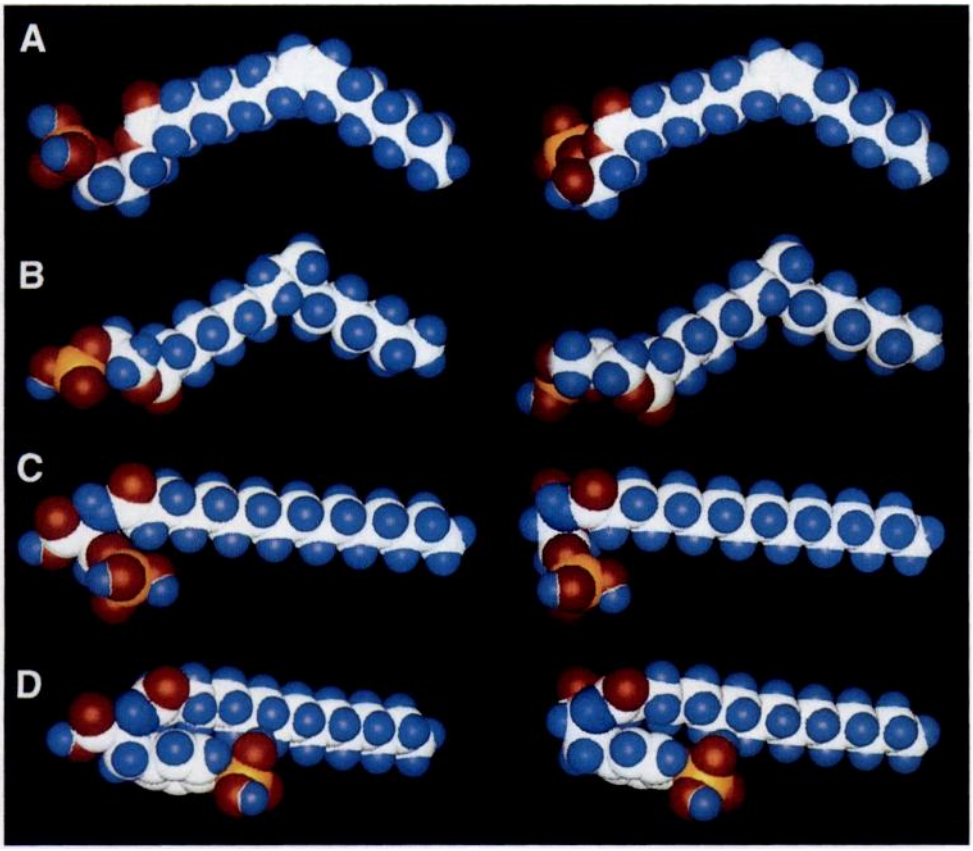


Fig. 2. Space-filling models of the molecules shown in Fig. 1, built with the SYBYL software based on the crystal structure of L-LPA. A, 18:1-LPA; B, cLPA; C, NP-Ser-PA; D, NP-Tyr-PA. *Left*, L-stereoisomers. *Right*, D-stereoisomers. Color codes of the atoms: *light blue*, hydrogen; *white*, carbon; *red*, oxygen; *dark blue*, nitrogen; and *orange*, phosphorus.

For electrophysiological measurements, agonists and antagonists were complexed with fatty acid-free bovine serum albumin at a 1:1 molar ratio in Ca^{2+}-free Ringer's solution at a concentration of 1 mM. Complexing of the compounds greatly improved the reproducibility of the inhibitory effect. Furthermore, the time needed for the oocytes to regain their sensitivity to LPA increased significantly in the absence of albumin complexation. These phenomena, which might be a consequence of a nonspecific hydrophobic interaction with the cell membrane lipids, were avoided completely by complexing the inhibitors with equimolar bovine serum albumin, as has been shown previously for the agonists (2, 3). For bath application, agonists and/or inhibitors were diluted directly in the superfusing solution and perfused at a velocity of 4–5 ml/min.

Intracellular microinjection of inhibitors. For intraoocyte application, NP-Ser-PA and NP-Tyr-PA were dissolved in dimethylsulfoxide at a concentration of 1 mM and filtered through a 0.45-μm membrane filter. Microinjection was achieved by pressure ejection from a glass micropipette at 420 kPa using a PicoPump (model PV 800; World Precision Instruments, New Haven, CT). Injection volume was calibrated by measuring the diameter of the drop at the tip of the micropipette. Intracellular concentrations of inhibitors were calculated on the basis of the cell diameter measured before introduction of electrodes.

Preparation and expression of poly(A)$^+$ mRNA from rat brain. Total mRNA was prepared from adult rat brain cortex according to the method of Chomczynski and Sacchi (28). Poly(A)$^+$ mRNA was isolated by chromatography on oligo(dT)-cellulose; 50 ng of poly(A)$^+$ mRNA was injected per oocyte. Different neurotransmitters and neuromodulators activating oscillatory Cl^- currents in the oocyte through a common second messenger system (29) to that activated by the LPA receptors were applied. Responses were recorded 5 days after the injection of mRNA and for ≥2 days after defolliculation with collagenase.

Mathematical analysis of the pharmacological data. EC_{50} and IC_{50} values were calculated by a nonlinear curve-fitting procedure using the Levenberg-Marquard algorithm. First, a single hyperbolic saturation curve was chosen as a model function, then a sum of two hyperbolic saturation curves, and so on. A model was accepted as best fit if 1) its χ^2 value was less than the χ^2 statistic value at the given degree of freedom at the 95% confidence level, 2) the mean value of the residuals did not differ significantly from zero at the 95% confidence level when examined by Student's t test, and 3) the number of the estimated binding sites was the least among models giving a fit satisfying the first and second requirements. The same approach was undertaken to fit model functions to all of the inhibition data (compare Figs. 3 and 5). In the mathematical treatment of data from experiments in which LPA was the agonist, in recognition of the two LPA receptors, a constraint for the fractional amounts of the two independent receptor sites was introduced and used uniformly. The ratio of the expression of the two LPA receptors was calculated by minimizing the sum of the χ^2 values extracted from the individual curve fits. All error values are presented at the 95% level of confidence.

Results

Effects of NP-Ser-PA and NP-Tyr-PA on currents elicited by LPA, a nonselective type I and II receptor agonist. Neither NP-Ser-PA nor NP-Tyr-PA nor their D-stereoisomers activated currents in the oocyte when used in concentrations of ≤10 μM (data not shown). To characterize the putative inhibitory activity of NP-Ser-PA and NP-Tyr-PA (see Figs. 1 and 2 for structures), dose-response relationships were established for LPA in the absence and in the presence of antagonists. A typical dose-response curve between the peak current amplitude and the concentration of 18:1-LPA in the superfusate is shown in Fig. 3. The threshold concentration for 18:1-LPA is typically between 0.1 and 1 nM, whereas

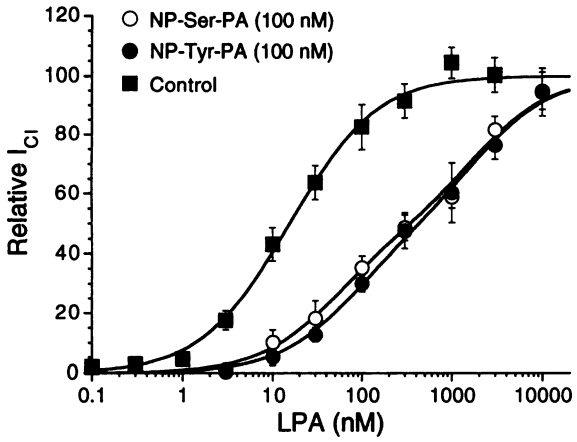


Fig. 3. Effects of NP-Ser-PA and NP-Tyr-PA on the dose-response relationship of the LPA response. Data represent normalized Cl^- current (I_{Cl}) (mean ± standard error of three or four experiments) activated by 18:1-LPA alone or mixed with one of the inhibitors (100 nM). Saturation curves were fitted to the data assuming two independent binding sites for LPA. Note the right shift in the dose-response curve caused by NP-Ser-PA and NP-Tyr-PA. Calculated EC_{50} values are listed in Table 1.

the saturating concentration is ~1 μM. As depicted in Fig. 3, in the presence of 100 nM NP-Ser-PA or NP-Tyr-PA, the dose-response curve of 18:1-LPA is shifted to the right by >1 order of magnitude. The shape of the dose-response curve was also altered; however, above the micromolar range of the agonist concentration, the dose-response curves reached the same maximal current, suggesting a competitive type of inhibition.

Mathematical analysis of these dose-response curves (Fig. 3) did not give a satisfactory fit to the data, assuming an interaction between the ligand and only one receptor site. In contrast, assuming two independent binding sites for 18:1-LPA, data could be approximated by nonlinear curve-fitting using the Levenberg-Marquard algorithm. The EC_{50} values yielded by the algorithm for 18:1-LPA in the absence of inhibitors and in the presence of 100 nM NP-Ser-PA or 100 nM NP-Tyr-PA are shown in Table 1. The fractional ratio of the two receptor sites was used as a fixed parameter in calculating of the individual curve fits, and this ratio was then optimized by minimizing the sum of χ^2 values. The optimization yielded a 48–52% ratio for high versus the low affinity receptor, which was used in all calculations (Figs. 3–5).

In the next set of competition experiments, 18:1-LPA was applied repeatedly close to its EC_{50} at a concentration of 10 nM, whereas the concentrations of the inhibitors were increased from 0.1 to 3000 nM. Twenty-minute intervals were allowed to elapse between consecutive applications to avoid errors due to homologous desensitization of the receptors. Twenty minutes has previously proved to be sufficient to allow the recovery of the oocyte from desensitization at this low concentration of the agonist (20). Fig. 4 shows the inhibition by NP-Ser-PA and NP-Tyr-PA of currents elicited with 10 nM 18:1-LPA. In the low concentration range, between 0.1 and 10 nM, both compounds showed similar inhibitory potency. However, at concentrations of >30 nM, NP-Ser-PA was less effective at inhibiting the currents, as demonstrated by a parallel shift of the inhibition curve to the left. Consistent with a competitive type of inhibition, both compounds reached a complete functional inhibition of the 18:1-LPA response in the micromolar range.

To quantify the inhibitory effects of NP-Ser-PA and NP-Tyr-PA displacement, curves were fitted to the data presented in Fig. 4. Again, a statistically confident fit could be achieved only if at least two independent binding sites for 18:1-LPA and a competitive mechanism of action were assumed. The calculated IC_{50} values for NP-Ser-PA and NP-Tyr-PA, with regard to the two LPA receptor sites, are shown in Table 2. The difference between the IC_{50} values of NP-

TABLE 1

EC_{50} values of the oscillatory responses elicited by LPA in the presence of 100 nM NP-Ser-PA and NP-Tyr-PA

Ligand	EC_{50}	
	High affinity site	Low affinity site
	nM	
LPA	7.0 ± 1.9	33 ± 8
NP-Ser-PA	49 ± 8	1880 ± 280
NP-Tyr-PA	76 ± 10	1990 ± 240

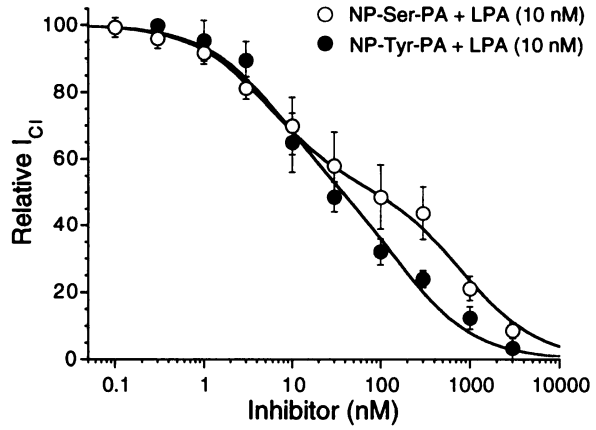


Fig. 4. Dose-response relationship of the inhibition of the LPA response by NP-Ser-PA and NP-Tyr-PA. LPA (10 nM) and one of the inhibitors were applied together. Curves were fitted assuming two independent binding sites for 18:1-LPA and a competitive type of inhibition. Calculated IC_{50} values are listed in Table 2. I_{Cl}, normalized Cl^- current.

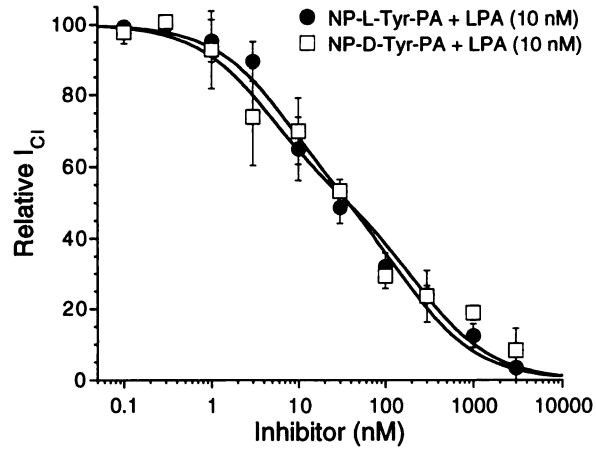


Fig. 5. Lack of stereoselectivity in the action of the inhibitors. To test the stereospecific action of the inhibitors, in addition to the L-stereoisomers, functional inhibition experiments were carried out with the D-stereoisomers of NP-Ser-PA and NP-Tyr-PA [normalized data of NP-L-Tyr-PA (five experiments) and NP-D-Tyr-PA (three experiments)]. I_{Cl}, normalized Cl^- current.

TABLE 2
IC_{50} values for NP-Ser-PA and NP-Tyr-PA against LPA and cLPA

Ligand	IC_{50}			
	High affinity site		Low affinity site	
	NP-Ser-PA	NP-Tyr-PA	NP-Ser-PA	NP-Tyr-PA
	nM			
LPA (10 nM)	5.4 ± 0.7	6.5 ± 1.5	805 ± 97	172 ± 36
cLPA (1.5 μM)			330 ± 30	490 ± 40

Ser-PA and NP-Tyr-PA for the low affinity site confirms the noticeable difference between the two inhibition curves in Fig. 4. Therefore, it seems that although the two inhibitors act equally well in blocking the high affinity site (type I receptor), NP-Tyr-PA is a marginally better inhibitor of the low affinity site (type II receptor).

Lack of stereoselectivity in the action of the inhibitors. Jalink *et al.* (21) reported that both L- and D-stereoisomers of LPA were active in eliciting Ca^{2+} transients in A431 cells, indicating the lack of stereospecificity of the receptors expressed in this cell line. To test the stereospecific action of the inhibitors, in addition to the L-stereoisomers, functional inhibition experiments were carried out with the D-stereoisomers of NP-Ser-PA and NP-Tyr-PA in oocytes. No significant differences between the inhibitory potencies of the L-and D-stereoisomers were found. The functional inhibition experiments for the L- and D-NP-Tyr-PA are shown in Fig. 5. The calculated IC_{50} values for the L- versus the D-isomer of NP-Tyr-PA were 6.5 ± 1.5 versus 4.4 ± 1.5 nM at the high affinity site and 172 ± 36 versus 232 ± 70 nM at the low affinity site. A similar agreement was reached in the case of NP-Ser-PA (data not shown).

Double reciprocal plot (Lineweaver-Burke) analysis of the pharmacological data. To validate the competitive mechanism of action of the inhibitors, double reciprocal plots of the dose-response data presented in Fig. 3 were generated (Fig. 6). The plots revealed two important features of the antagonists. First, the dose-response data were nonlinear, indicating that more than one receptor is involved in the response to 18:1-LPA. Second, all dose-response curves measured in the presence and absence of the inhibitors intercepted the ordinate at 1, fulfilling the requirement of a competitive mechanism of action.

Effects of NP-Ser-PA and NP-Tyr-PA on currents elicited by cLPA, a type II receptor agonist. In the next set of functional inhibition experiments, cLPA was chosen as the agonist at a concentration of 1.5 μM, which is its approximate EC_{50} value, and the concentrations of the antagonists were varied between 10 and 3000 nM. Representative functional inhibition curves of NP-Ser-PA and NP-Tyr-PA are illustrated in Fig. 7. NP-Ser-PA showed a slightly higher potency over NP-Tyr-PA in competition for the cLPA receptor site. These data sets could be satisfactorily fitted by a displacement curve, assuming only one site of action with a competitive mechanism of inhibition. The IC_{50} values calculated for NP-Ser-PA and NP-Tyr-PA against cLPA on the low affinity receptor site are shown in Table 2.

NP-Ser-PA and NP-Tyr-PA act on extracellular sites. To map the site of inhibition, the compounds were also introduced through intracellular microinjection into the oocyte. Fig. 8 shows a representative experiment in which NP-Ser-PA was first applied extracellularly and then injected intracellularly from a micropipette. The injection pipette was filled with 1 mM inhibitor and introduced into the oocyte. The injection pressure was set so that an estimated intracellular concentration of 100 nM of the inhibitor was achieved. As shown in Fig. 8, 100 nM NP-Ser-PA applied in the superfusate reduced the response to 18:1-LPA by 64% of the control, whereas it had no effect when injected intracellularly. Experiments carried out with NP-Tyr-PA gave similar results (data not shown).

NP-Ser-PA and NP-Tyr-PA do not interfere with other receptors that activate phosphoinositide breakdown, Ca^{2+} release, and oscillatory Cl^- currents in the oocyte. To rule out a nonspecific action of the inhibitors on elements of the signal transduction machinery involved in the generation of oscillatory Cl^- currents, the effects of NP-Ser-PA and NP-Tyr-PA were tested on different neurotransmitter/neuromodulator receptors expressed heterologously in the oocyte. Oocytes were injected with 50 ng/cell rat cortex

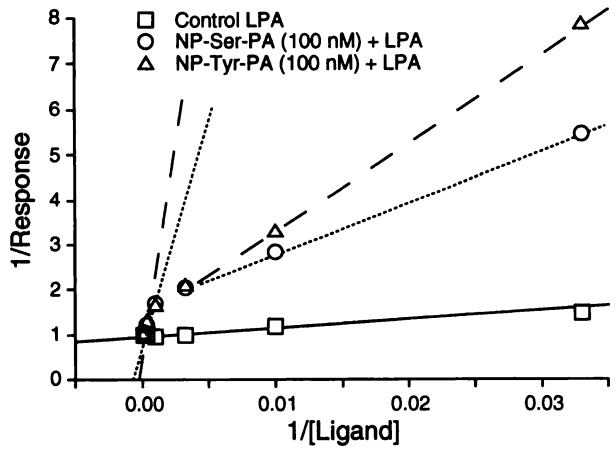


Fig. 6. Double-reciprocal plot of the inhibition of the LPA response by NP-Ser-PA and NP-Tyr-PA. Lineweaver-Burke analysis confirms a nonlinear dose-response relationship for LPA approximated by two linear functions (*dotted lines*, NP-Ser-PA; *dashed lines*, NP-Tyr-PA). Note that all curves intercept the ordinate at 1, which is consistent with a competitive mechanism of action.

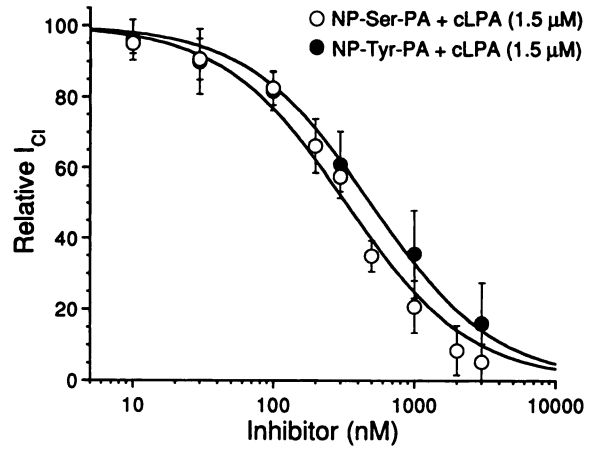


Fig. 7. Inhibition of the cLPA receptor by NP-Ser-PA and NP-Tyr-PA. Currents elicited by 1.5 μM cLPA were inhibited by the coapplication of NP-Ser-PA and NP-Tyr-PA. Normalized data sets (mean ± standard error of four experiments) were fitted with a single curve, assuming only one binding site and a competitive mechanism of inhibition. Calculated IC_{50} values are given in Table 2. I_{Cl}, normalized Cl^- current.

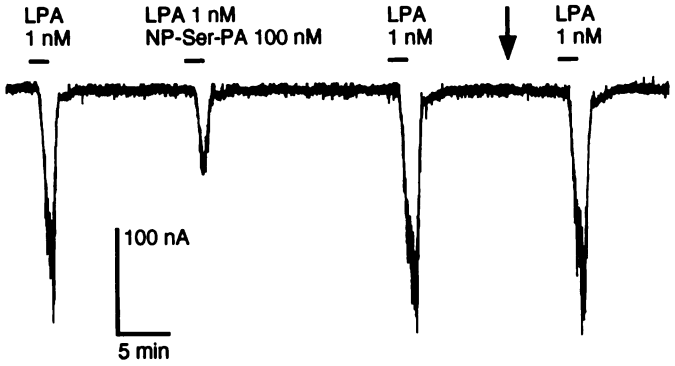


Fig. 8. Intracellular application of NP-Ser-PA does not affect the LPA response. In this experiment, LPA was applied at a concentration of 1 nM. When the inhibitor was applied extracellularly together with the agonist, it caused a ~65% inhibition of the current. In contrast, microinjection of the antagonist (*arrow*) into the oocyte from a glass micropipette filled with 1 mM NP-Ser-PA, which reached an estimated intracellular concentration of 100 nM, had no detectable effect on the response.

poly(A)$^+$ mRNA, and responses to acetylcholine, serotonin, glutamate, and kainate were measured 5 days after injection. With the exception of kainate, which acts on a receptor that is an intrinsic receptor/Cl^- channel molecule, the other neurotransmitters and neuromodulators elicit oscillatory Cl^- currents in the oocyte via a common signal transduction pathway. Responses elicited by concentrations of these different neurotransmitters/neuromodulators, close to their EC_{50} values, obtained in the presence or absence of 1 μM NP-Ser-PA or NP-Tyr-PA are presented in Table 3. Representative current traces for the application of serotonin, kainate, and 18:1-LPA (for comparison) measured in the same cell are shown in Fig. 9. No significant differences were found between responses, with the exception of the LPA response measured in controls treated with vehicle only and those treated with LPA inhibitors. Lysophosphatidyl-*sn*-glycerol, which was reported to attenuate the binding of a photoaffinity-label derivative of LPA (12) and LPA-induced Ca^{2+} responses (22), exhibited neither agonist nor antagonist activity when applied at ≤10 μM in the oocyte assay system (data not shown).

Discussion

LPA is a lipid mediator with diverse actions, ranging from growth factor-like activities to the induction of chemotactic motility in cells; its origin spans the phylogenetic tree from *Dictyostelium* to humans (1). There is a consensus that LPA

TABLE 3

NP-Ser-PA and NP-Tyr-PA do not interfere with other receptors eliciting Cl^- currents in oocytes

Electrophysiological recording was carried out 5 days after injection with rat cortex poly(A)$^+$ mRNA. NP-Ser-PA or NP-Tyr-PA (1 μM) was coapplied with the different agonists. Responses (mean ± standard error for three to five experiments) are given in percentages normalized to control (agonists without the inhibitors).

	NP-Ser-PA	NP-Tyr-PA
	%	
Acetylcholine (10 μM)	108 ± 7	105 ± 6
Serotonin (1 μM)	104 ± 4	107 ± 12
Glutamate (10 μM)	100 ± 3	93 ± 4
Kainate (100 μM)	97 ± 7	94 ± 11

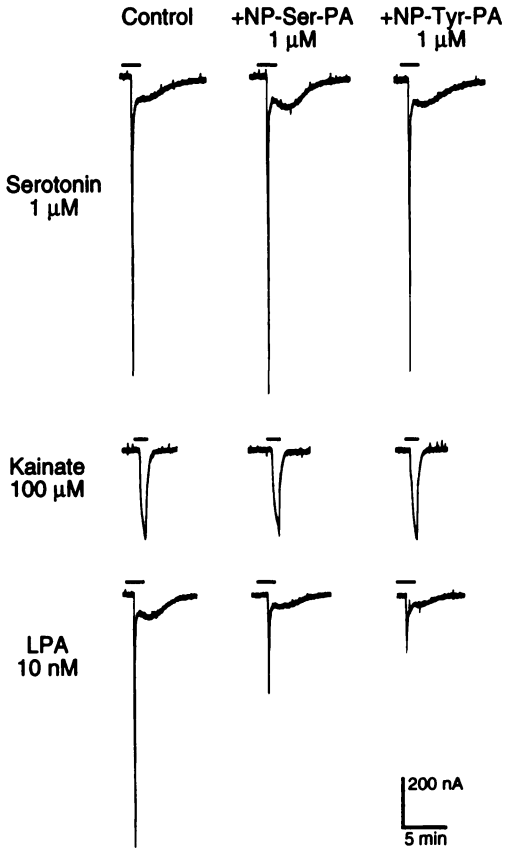


Fig. 9. NP-Ser-PA and NP-Tyr-PA do not interfere with serotonin- and kainate-elicited Cl^- currents. Traces were obtained from the same oocyte injected with rat brain mRNA. Serotonin and LPA activated Cl^- currents mediated by the phosphoinositide/Ca^{2+} second messenger system, whereas kainate activated a glutamate receptor subtype, which is an intrinsic receptor/channel complex. The inhibition of the response to LPA is also shown for comparison. Agonists alone (*first column*) or mixed with 1 μM NP-Ser-PA (*second column*) or NP-Tyr-PA (*third column*) were tested in a random sequence.

acts primarily as an extracellular mediator on a family of G protein-coupled plasma membrane receptors. LPA is not the only naturally occurring phospholipid that elicits this wide array of cellular responses but rather a member of a group of phospholipids that are generated from stressed, activated, or injured cells in both animals and plants. For example, in human serum, there are ~10 not fully characterized phospholipids with LPA-like activities that are generated during blood clotting, primarily from platelets (3). The overall understanding of the physiological role of the diverse and often opposing effects of these LPA-like lipid mediators would greatly benefit from the availability of selective and specific inhibitors. However, because of the very strict structure-activity relationship characterizing the LPA mediator family, the development of such inhibitors is not easy. Although the local anesthetic procaine blocks the action of LPA in the *X. laevis* oocyte (30), local anesthetics do not provide the selectivity necessary to separate their activities from those evoked by other phospholipid mediators having similar structures (e.g., PAF, sphingosine-1-phosphate, lysosphingomyelin). Previous reports have delineated the structure-activity relationship of LPA-like lipid mediators (3, 13, 16, 21) by establishing the following principles: 1) the structural requirement for a long hydrocarbon chain, of which 18:1-LPA was

found to be the most potent in many cases, whereas lauroyl- and decanoyl-LPA show little or no activity; 2) the covalent attachment of this nonpolar group to a phosphate group through a C—O—P-type structure because phosphonate analogs have no biological activity; and 3) the lack of stereospecificity at the *sn*-2 position of the glycerol backbone. All of these and results of other studies clearly point to the existence of receptors for LPA-like lipid phosphatidates, distinct from those for PAF, sphingosine-1-phosphate, and lysosphingomyelin. In human A431 cells, sphingosine-1-phosphate, which is similar to PAF, was not an agonist of the LPA receptor (21). Similar results were established in atrial myocytes (31).

In a search of possible agonists of the LPA receptor in human platelets, Sugiura *et al.* (16) observed that although *N*-palmitoyl-2-aminoethanol phosphoric acid caused the aggregation of platelets, other derivatives of this type (e.g., NP-Ser-PA, NP-Tyr-PA) exhibited inhibitory activity toward aggregation elicited by 16:0-GP. We chose NP-Ser-PA and NP-Tyr-PA as potential candidates for LPA antagonists and developed a novel method for their synthesis (24). To perform a detailed pharmacological analysis of their action, we used the *X. laevis* oocyte bioassay because of the several unique advantages offered by this system compared with platelet aggregation. In *X. laevis* oocyte, LPA activates a Ca^{2+}-dependent oscillatory Cl^- current (3, 6, 17–20). The structure-activity relationship derived from studies in oocytes matches that obtained in mammalian cells (21). The pharmacologically different LPA receptor subtypes have been best characterized in the oocyte (20). Another advantage in using the oocyte bioassay is that oocytes do not express receptors to PAF (3); despite the contradictory report on sphingosine-1-phosphate-elicited Cl^- currents in *X. laevis* oocytes (32), in our trials this agent was completely inactive in doses of ≤ 10 μM (20), whereas the response threshold to LPA is <1 nM.

Similar to LPA and cLPA, the inhibitors were without any effect when applied intracellularly at a high (100 nM) concentration (see Fig. 8). Consequently, we hypothesize that a competitive-type inhibition occurs at sites facing the extracellular surface, which are unique to the LPA receptors because the inhibitors did not affect oscillatory and smooth Cl^- currents elicited by a variety of other receptors. The fact that the inhibitors were without any effect on the oscillatory currents evoked by the various other receptors is important for yet another reason, indicating that they did not affect the receptor/channel coupling mechanism, commonly used by these and the LPA receptors.

On the basis of competition experiments (see Fig. 4), IC_{50} values for NP-Ser-PA and NP-Tyr-PA were found to be in the nanomolar range, rendering them potent antagonists. Although the two compounds did not show significant differences relative to each other in the 0.1–10 nM range, NP-Ser-PA proved to be a slightly weaker competitor for 18:1-LPA at the low affinity receptor site. However, we feel that this difference in potency between the two antagonist at the low affinity site is not sufficiently large to be used to achieve a receptor subtype-specific inhibition. When the inhibitors were applied in the micromolar range, they both completely diminished the response, further supporting the hypothesis of a competitive mechanism of action. Pharmacological analysis of the inhibition data using the Lineweaver-Burke plot (Fig. 6) corroborated the existence of at least two independent competition/receptor sites and is consistent with our recent report that there are at least two distinct receptors expressed for LPA in the oocyte (20). In contrast, the inhibition curve shown in Fig. 7 for the type II receptor subtype-specific agonist cLPA (20) could be satisfactorily fitted with one site of interaction, assuming a competitive mechanism of action.

The observation that there was no significant difference between inhibitory effects of the L- and D-stereoisomers of NP-Ser-PA and NP-Tyr-PA (see Fig. 5) is in agreement with the finding that both the L- and D-stereoisomers of lysophosphatidates are capable of activating their receptors (16, 21). Analysis of the space-filling molecular models of NP-Ser-PA and NP-Tyr-PA revealed some interesting common structural features. The structure of LPA showed a linear chain with a polar head group that looks like a hook (see Fig. 2A). In contrast, the inhibitors showed a hairpin-like structure in which the head group turns back on the acyl chain. This hairpin turn alters the distance of the phosphate moiety relative to the acyl chain, whose positions relative to each other seemed to be important for agonist activity (3, 21). The major difference between the L- and D-isomers appeared in the turn region, which because of the similar potencies of the isomers might not have an important role in interacting with the binding site.

An important question remains to be answered regarding the specificity of inhibition caused by NP-Ser-PA or NP-Tyr-PA. Sugiura *et al.* (16) reported that these inhibitors did not affect platelet aggregation induced by arachidonic acid, thrombin, or PAF. Oscillatory Cl^--currents evoked in the oocyte following the heterologous expression of the human PAF receptor show no cross-desensitization with the LPA response,[1] supporting that the two lipids act on distinct receptors. However, in the oocyte, possible further interactions with the signal transduction machinery involved in the mechanism of oscillatory currents could also be ruled out. Oocytes injected with poly(A)$^+$ mRNA purified from rat brain express different subtypes of functional neurotransmitter and neuromodulator receptors (29). Using this heterologous expression technique, we found no effects of the inhibitors in the acetylcholine, serotonin, glutamate, and kainate responses (see Table 3 and Fig. 9). Based on these findings, the inhibitory activities of NP-Ser-PA and NP-Tyr-PA are restricted to the disruption of the interaction between the LPA-like lipid mediators and their putative plasma membrane receptors.

The present data provide reasonable grounds for hypothesizing that NP-Ser-PA and NP-Tyr-PA are specific competitive inhibitors of the LPA receptors in the oocyte. These *N*-acyl-amino acid phosphate derivatives, particularly the tyrosine analog, are ideal targets for labeling with radioactive isotopes and thus might also prove helpful in the search for putative LPA receptors. Nevertheless, NP-Ser-PA and NP-Tyr-PA might already be useful in experiments to inhibit responses to LPA and can serve as prototypes for designing antagonists with even higher inhibitory potency and/or higher selectivity for the different LPA receptor subtypes. The availability of receptor subtype-selective inhibitors would be extremely useful in understanding how the distinct LPA receptors function *in vivo*.

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