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ACCELERATED COMMUNICATION

Characterization of a Receptor Subtype-Selective Lysophosphatidic Acid Mimetic

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ABSTRACT

Despite an intriguing cell biology and the suggestion of a role in pathophysiological responses, the mechanism of action of such lipid phosphoric acid mediators as lysophosphatidic acid (LPA) remains obscure, in part because of an underdeveloped medicinal chemistry. We report now the agonist activity of a synthetic phospholipid in which the glycerol backbone of LPA is replaced by L-serine. Like LPA, the L-serine-based lipid mobilizes calcium and inhibits activation of adenylyl cyclase in the human breast cancer cell line MDA MB231. Treatment with LPA desensitizes MDA MB231 cells to subsequent application of the L-serine compound; when the order of application is reversed, however, the L-serine compound does not prevent calcium mobilization by LPA, which might indicate the existence of two LPA receptors in these cells. The analogous D-serine-based phospholipid was distinctly less potent than the L-isomer in those assays; this finding demonstrates stereoselectivity by an LPA receptor. Unlike LPA, the L-serine-based lipid does not evoke a chloride conductance in *Xenopus laevis* oocytes, but injection of poly(A)$^+$ RNA from HEK 293 cells confers this phenotype on the oocyte. The latter result has practical importance in that it allows use of the frog oocyte for expression cloning of an LPA receptor DNA, an assay system made problematic by the oocyte's strong endogenous response to LPA.

The ability of LPA to mediate a wide range of responses in many cell types has been demonstrated repeatedly during the past decade. In a variety of cultured cells, these responses include calcium mobilization (Jalink *et al.*, 1990), inhibition of adenylyl cyclase activation (van Corven *et al.*, 1992), and formation of focal adhesions (Ridley and Hall, 1992). Less is known about LPA's effect on tissues, but LPA has been shown to induce platelet aggregation (Watson *et al.*, 1985), smooth muscle contraction (Tokumura *et al.*, 1991; Tokumura *et al.*, 1980) and skin thickening (Piazza *et al.*, 1995). It is assumed widely that LPA signals cells at least in part through one or more G protein-coupled receptors.

Although there exists a well-developed cell biology and a good understanding of intracellular signaling events in cultured cells after LPA activation, our understanding of LPA's interaction with its receptor(s) is primitive. This is partly because of a lack of LPA-based medicinal chemistry, including LPA mimetics and receptor antagonists. Sugiura *et al.* (1994) synthesized a series of lipid phosphoric acids and investigated their effects on aggregation of human platelets. Among the compounds described in their seminal paper (Sugiura *et al.*, 1994) were *N*-palmitoyl serine phosphoric acid (NASPA) and *N*-palmitoyl tyrosine phosphoric acid, both of which they found to act as insurmountable antagonists of LPA-induced platelet aggregation. Subsequently, Liliom *et al.* (1996a) synthesized the same compounds and demonstrated that both act as competitive antagonists of the chloride conductance elicited when LPA is applied to the surface of *Xenopus laevis* oocytes. They synthesized both stereoisomers and found them to be equipotent.

In light of the blocking activity ascribed to these phosphoamino-acid-based compounds, we considered using them as

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ABBREVIATIONS: LPA, lysophosphatidic acid; NOEPA, *N*-oleoyl ethyl phosphoric acid; NASPA, *N*-acyl serine phosphoric acid; TRAP, thrombin receptor activating peptide; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; HEK, human embryonic kidney; $I_{Cl(Ca)}$, calcium-induced chloride current; EDG-2, endothelin differentiation gene-2.

lead structures that might, through subsequent synthetic manipulation, be optimized to develop a high affinity LPA receptor antagonist for a mammalian LPA receptor. Concentrating on the serine-based compound, we duplicated readily the synthetic route and antagonist activity on frog oocytes reported by Liliom et al. (1996a) and Bittman et al. (1996). To our surprise, however, we found that L-NASPA is a potent *agonist* on cultured mammalian cells with regard to both calcium mobilization and inhibition of adenylyl cyclase activation, whereas D-NASPA is less potent in these assays. With this article, we present the results of our characterization of these compounds.

Materials and Methods

Compound synthesis and purification. Synthesis of the D- and L- isomers of NASPA was conducted exactly as described by Bittman *et al.* (1996), except that we substituted 30% hydrogen peroxide (aqueous) for 3-chloroperoxybenzoic acid in oxidizing the phosphite intermediate. All reactions were carried out under inert atmosphere, all solvents were purified by filtration through alumina (activity I) and the reaction products were purified on 230–400 mesh silica gel.

The purities of both D- and L-NASPA were determined using normal phase HPLC with an analytical Microsorb-MV column (4.6 × 250 mm; Rainin/Varian Instruments, Walnut Creek, CA) packed with 5-μm silica (1000-nm pore size). A Waters two-pump gradient system was used with a flow rate of 1 ml/min. The column effluent was monitored with an evaporative light scattering detector (Alltech Model 500; Alltech Associates, Inc., Deerfield, IL). The solvent system consisted of solvent A [composed of $HCCl_3$/MeOH/NH_4OH (30%) in a ratio of 80:19.5:0.5] and solvent B [composed of $HCCl_3$/MeOH/H_2O/NH_4OH (30%) in a ratio of 34.5:55:10:0.5].

The samples were prepared for HPLC in 1:1 chloroform/methanol at 5 mM concentrations. Injections of 10 μl were made with a U6K injector (Waters, Milford, MA) and eluted over an isocratic gradient 90:10 A/B over 5 min and a linear gradient to 30:70 A/B over 10 min followed by isocratic application of 30:70 A/B for 30 min. The detector evaporator tube was set to 92°, the exhaust tube to 52°, and the nitrogen gas flow was set to 2.45 standard liters per minute. The retention times of both the pure D- and L-NASPAs were 33 min under these conditions.

Compound preparation. Compounds were quantified by colorimetric phosphate analysis (Kingsley and Feigenson, 1979) and freed of organic solvents in an evacuated microcentrifuge (SpeedVac; Savant Instruments, Farmingdale, NY). The compounds were dissolved in water containing 0.1% fatty acid-free BSA and serially diluted in the same solvent. To manipulate solutions containing large concentrations (≥ 100 μM) of compounds, we used water containing 1.0% fatty acid-free BSA.

Cell culture. Lipid phosphoric acids were tested on the human breast cancer cell line MDA MB231. This cell line was chosen to test compounds generated in our synthetic program because it is both highly and reproducibly responsive to LPA; also, these cells are simple to grow. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and were passaged after trypsinization every 5–7 days. Their growth medium was changed every third day. Our compounds were tested as well, albeit less extensively, on the adenovirus-transformed HEK 293 cell line and on the LPA-nonresponsive human myeloid cell line, K562. These cells were cultured in 1:1 Dulbecco's modified Eagle's medium/Ham's F12 medium and RPMI 1640 medium, respectively. The growth media for all cell lines were supplemented with 10% fetal bovine serum and the cultures were kept at 37° in a humidified atmosphere containing 5% CO_2.

Calcium and cAMP assays. Calcium mobilization and cAMP assays were performed as we described previously (Lynch *et al.*, 1997). Briefly, intracellular calcium fluxes were measured on cell populations (2–4 × 10^6 cells) that had been loaded with the calcium sensitive fluorophore INDO-1. Responses, which were measured in a temperature controlled fluorimeter (Aminco SLM 8000C; SLM Instruments, Urbana, IL), are reported as the fraction of the maximal response (i.e., response to 75 μM digitonin). The BSA vehicle was determined to have no response. Assays of adenylyl cyclase activity were conducted on populations of 5 × 10^4 cells stimulated with 10 μM forskolin in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine. cAMP was measured by automated radioimmunoassay.

Oocyte preparation and recording. Oocytes were obtained from *X. laevis* frogs. Surgical removal and defolliculation of oocytes for injection and electrophysiological recording were performed as we described previously (Krieger and Hook, 1992; Durieux *et al.*, 1993). The animal use protocol was approved by the Animal Research Committee at the University of Virginia. Total RNA was prepared from cultured cells according to the method of Chomczynski and Sacchi (1988) and the poly(A)$^+$ RNA fraction was enriched by chromatography on oligo(dT) cellulose (Aviv and Leder, 1972). Oocytes were injected with 30 nl of sterile water containing 0.4 ng/nl poly(A)$^+$ RNA using an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA). The cells were then incubated at 18° for 96 hr before study.

Electrophysiological recording. Records were made from single defolliculated oocytes held in a continuous flow recording chamber. The cell was voltage-clamped using a two-microelectrode oocyte voltage clamp amplifier (OC725A; Warner Corporation, New Haven, CT) and only cells exhibiting stable holding currents of less than 1 μA during a one minute equilibration period were included in the analyses. Compounds were delivered as a 30-μl aliquot over a period of 1–2 sec using a hand-held micropipette positioned approximately 3 mm from the oocyte surface. Responses were quantified by integrating the current trace and are reported as mean ± standard error in microcoulombs. Differences among treatment groups were analyzed using Student's t test or Mann-Whitney U test. If multiple comparisons were made, data were analyzed using analysis of variance followed by t test corrected for multiple comparisons (Bonferroni) or Kruskal-Wallis one-way analysis of variance on ranks. Significance was reached at $p < 0.05$.

Construction of EDG-2 cDNA expression plasmid. We obtained the EDG-2 orphan G protein-coupled receptor cDNA from the I.M.A.G.E. Consortium (Lennon and Auffray, 1996) via Research Genetics (Birmingham, AL). This cDNA (clone ID# 515216) is from mouse testis and our DNA sequence analysis showed that it contains a full translational open reading frame. The open reading frame was amplified using the polymerase chain reaction (primers: forward 5′-gcc tct aga ggt acc atg gca gct gcc tc, reverse 3′-gcc gga tcc tcg agc taa acc aca gag tgg tc) and, after restriction endonuclease digestion with *Kpn*I and *Xho*I, subcloned into the same sites of the eukaryotic expression plasmid, pcDNA-3. The identity of the final plasmid construct was confirmed by automated DNA sequence analysis of the 1.05 kilobase-pair cDNA insert. [This plasmid has been deposited with the American Type Culture Collection (Rockville, MD).]

Materials. L-serine was purchased from Sigma (St. Louis, MO) and its D-isomer was purchased from Bachem (Torrance, CA). Palmitoyl chloride was purchased from Sigma, dibenzyl diisopropylphosphoramidite was purchased from Aldrich (Milwaukee, WI) and LPA (1-oleoyl) was purchased from Avanti Polar Lipids (Alabaster, AL). Frogs were purchased from Xenopus I (Ann Arbor, MI). Solvents for HPLC analyses were purchased from J. T. Baker (Phillipsburg, NJ). DNA sequence analyses and synthesis of the PAR-1 TRAP (SFLLR-Namide) were performed in the University of Virginia's Biomolecular Research Facility. The pcDNA-3 plasmid was from InVitrogen (Carlsbad, CA). Pertussis toxin was a gift from Dr. Erik Hewlett of the Department of Internal Medicine at University of Virginia. All other chemicals were from Sigma.

Results

We concentrated our efforts on the serine-based lipid phosphoric acid for two reasons. First, we have shown previously that a structural analog, NOEPA, is a potent, full LPA mimetic (Lynch *et al.*, 1997) (structures and IUPAC nomenclature presented in Fig. 1) and wished to explore further structural variations on this ethanolamine-based compound. Second, we encountered considerable difficulties regarding the aqueous solubility of the tyrosine-containing phospholipid; this problem reduced the reproducibility of effects observed with this compound.

On the basis of reports that NASPA antagonizes LPA-induced human platelet aggregation (Sugiura *et al.*, 1994) and amphibian oocyte depolarization (Liliom *et al.*, 1996a), we hypothesized that this compound would similarly block LPA-induced calcium transients in mammalian cell lines such as MDA MB231 and HEK 293. However, we disproved this hypothesis with our initial experiments, in which we found that L-NASPA evoked strong calcium signals when applied to these cells in nanomolar concentrations. The dose-response relationships for both LPA and the L- and D-isomers of the serine-based compound on MDA MB231 cells are presented in Fig. 2A. The L-isomer was equipotent to LPA in this assay (EC_{50}, 5.3 nM versus 5.6 nM), but its maximal efficacy was only about two thirds of that of LPA. In contrast, the D-isomer of NASPA exhibited a drastically lower potency and maximal efficacy. Using D-NASPA, no response was detected below 100 nM—a concentration that is about two log orders of magnitude greater than the EC_{50} value for the L-isomer. At the highest dose applied to the cells (10 μM), D-NASPA induced mobilization of only 5–10% of available calcium. L-NASPA induced strong calcium transients at comparable concentrations in HEK 293 cells as well (not shown).

In view of this discovery, we explored the agonist properties of L-NASPA further. If L-NASPA were a full LPA mimetic [as are ethanolamine-based lipid phosphoric acids (Lynch *et al.*, 1997)], one expects full, bidirectional cross-desensitization with LPA-evoked responses. However, we found that while prior LPA application prevents calcium transients in response to subsequent L-NASPA application, the converse was not so (Fig. 3). The same pattern of unidirectional desensitization was also observed in HEK 293 cells (data not shown). The ethanolamine-based lipid, NOEPA, behaved exactly as LPA did in this assay, whereas sphingosine-1-phosphate did not cross-desensitize responses to LPA, NASPA, or NOEPA (data not shown). The downstream stimulus-response mechanism remained intact regardless of the order of addition of the lipid phosphate compounds, because subsequent stimulation of the PAR-1 thrombin receptor (using the thrombin mimetic TRAP) evoked its normal, large calcium transient.

A second, nearly invariant aspect of LPA signaling that accompanies calcium mobilization is the inhibition of adenylyl cyclase stimulation. The data presented in Fig. 2B demonstrates that L-NASPA and LPA are again nearly equipotent (EC_{50} values were 25 nM and 52 nM, respectively), and L-NASPA is fully effective in suppressing forskolin-driven increases in cAMP levels. D-NASPA is about one log order of magnitude less potent in this assay (EC_{50} = 350 nM) but is clearly fully effective at suppressing adenylyl cyclase activation.

Among the strongest evidence for the idea that LPA acts through a heterotrimeric G protein signaling system is the elimination of some LPA responses by pertussis toxin. To learn whether inhibition of adenylyl cyclase by L-NASPA is blocked by the toxin, we treated cultures of MDA MB231 cells with 100 ng/ml of pertussis toxin for 16 hr. At a concentration of 1 μM, LPA and L-NASPA inhibited cyclase activity, but prior treatment with the toxin eliminated this inhibition (Fig. 4).

In addition, we tested L-NASPA for its ability to evoke calcium transients in K562 cells, a human myeloid cell line that we have found on repeated testing to be entirely nonresponsive (calcium mobilization and inhibition of adenylyl cyclase) to lipid phosphoric acid mediators including LPA, NOEPA, and sphingosine-1-phosphate. These cells did not

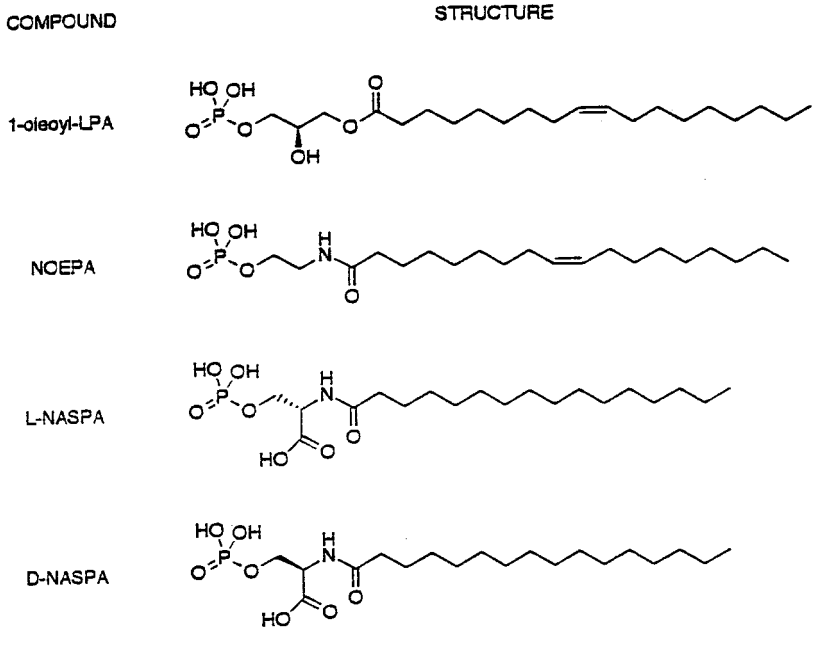

Fig. 1. Structural representation of glycerol-, ethanolamine- and serine-based lipid phosphate mediators: the compounds are LPA (1-oleoyl lysophosphatidic acid), NOEPA, and NASPA. The IUPAC nomenclature is: 2(*S*)-hydroxy-3-(phosphonoxy)propyl-(*z*)-9-octadecenoate; 2-[(*z*)- 9-octadecenoylamino]ethyl dihydrogen phosphate; 2-L-(palmitoylamino)-3-(phosphonoxy)propionic acid and 2-D-(palmitoylamino)-3-(phosphonoxy)propionic acid, respectively.

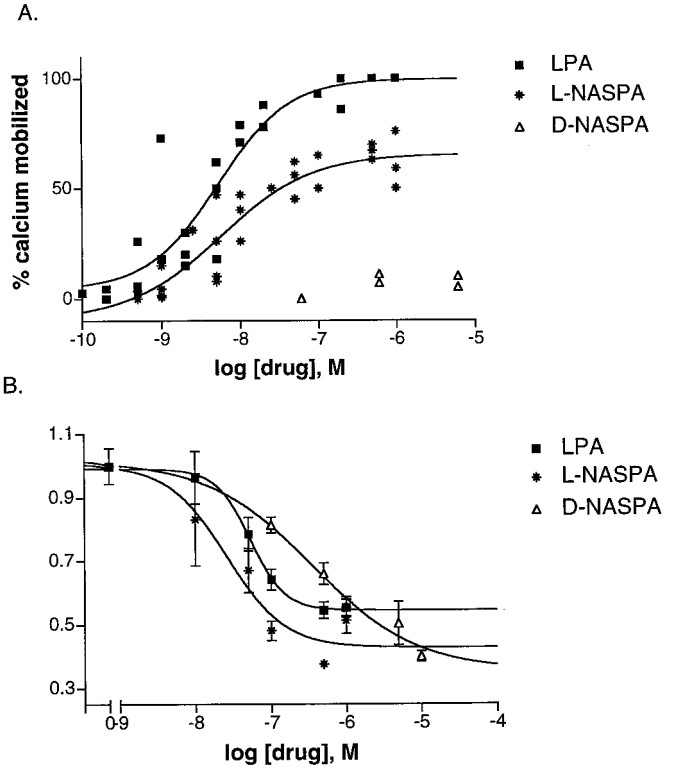

Fig. 2. Dose-response curves for calcium mobilization and inhibition of adenylyl cyclase activity by LPA, L-NASPA, and D-NASPA. A, Calcium mobilization was measured as a function of INDO-1AM fluorescence and is presented as a percent of the total available calcium (defined by subsequent treatment with digitonin). *Points,* from an individual record of experiments on two separate days; *curve*, fitted using GraphPAD (GraphPAD Software, San Diego, CA) (non-linear regression, sigmoidal dose response). The EC_{50} values from these curves are 5.3 nM (LPA) and 5.6 nM (L-NASPA). No EC_{50} was determined for D-NASPA. B, Inhibition of adenylyl cyclase activity by LPA, L-NASPA and D-NASPA. MDA MB231 cells populations (5×10^4 cells/assay tube) were treated with forskolin (10 μM) and isobutylmethylxanthine (1 mM) and series of LPA and NASPA concentrations. *Points,* in triplicate; *curve,* fitted using GraphPAD (non-linear regression, sigmoidal dose response) The calculated EC_{50} values are: 52 nM (LPA), 25 nM (L-NASPA), and 350 nM (D-NASPA). A relative value of 1.0 represents untreated cells (0.1% BSA added instead of drug), with an average value of 18 pmol of cAMP per 3.75×10^5 cells.

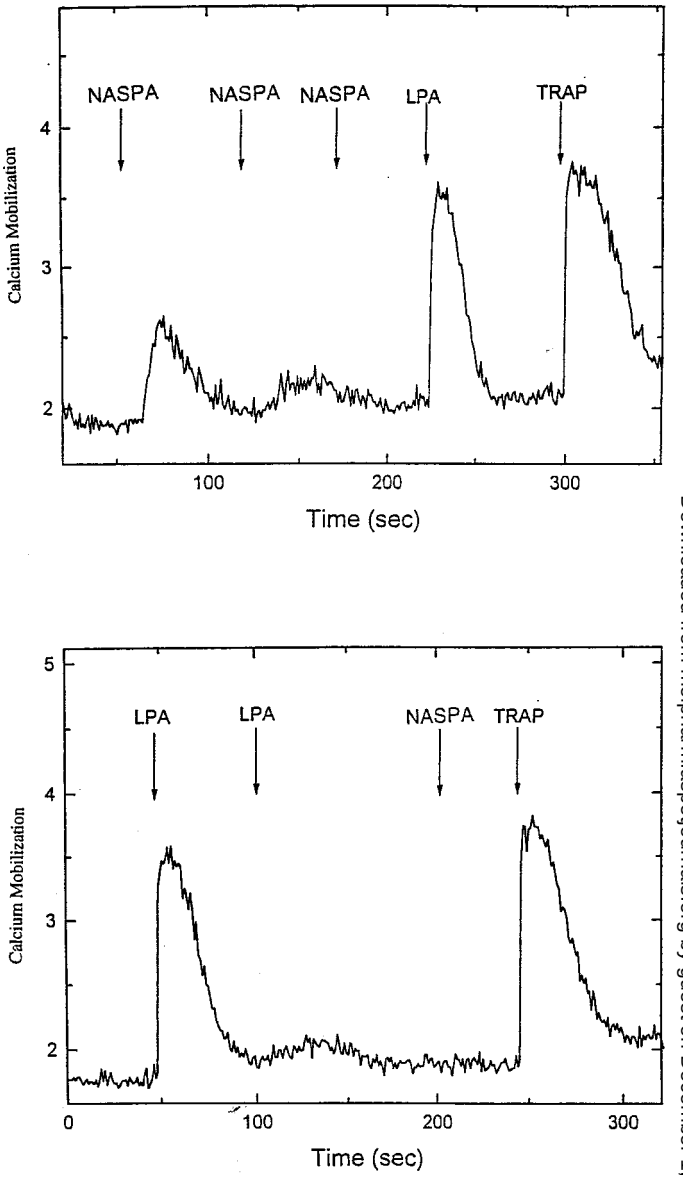

Fig. 3. Cross-desensitization of LPA and L-NASPA. A population of MDA MB231 cells ($2–4 \times 10^6$ cells/cuvette) that was desensitized by repeated maximal doses of LPA (1 μM) did not respond to an additional, maximal dose (1 μM) of L-NASPA. Further application of TRAP (SFLLR-Namide) to 100 μM demonstrated that this stimulus-response mechanism remained intact. When the phosphate compounds were applied in reverse order (i.e., several maximal doses of L-NASPA followed by 1 μM LPA), the latter compound evoked a full calcium signal, as did subsequent 100 μM TRAP.

show calcium mobilization to even the highest dose (10 μM) of L-NASPA, although they exhibited their characteristic robust responses to TRAP (100 μM) and the thromboxane A_2 mimetic, U46619 (1 μM) (data not shown).

X. laevis oocytes are known to have a strong, consistent response (i.e., depolarization) to applied LPA (Tigyi *et al.*, 1991; Durieux *et al.*, 1992; Fernhout *et al.*, 1992; Ferguson and Hanley, 1992) and the LPA mimetic NOEPA (Lynch *et al.*, 1997). Because our results with L-NASPA contradict the reported competitive antagonism of LPA's effect on frog oocytes (Liliom *et al.*, 1996a), we tested L-NASPA for antagonist activity in the oocyte system. We confirmed the results of Liliom *et al.,* in that the dose-response curve for LPA exhibited a dextral shift in the presence of 1 μM L- or D-NASPA (Hönemann CW and Durieux ME, unpublished observations, 1997). The endogenous response to LPA makes the use of the oocyte as an expression cloning tool difficult, but the apparent selectivity of L-NASPA for the mammalian response presents an opportunity to regain this assay system. To test this concept, we injected poly(A)$^+$ RNA extracted from HEK 293 cells (injection of MDA MB231 poly(A)$^+$ RNA kills oocytes) and, after 4 days, recorded responses to L-NASPA. As shown in Fig. 5, robust inward current responses were observed when L-NASPA was applied to oocytes injected with RNA from HEK 293 cells.

A practical aspect of our findings is that the molecular cloning of an LPA receptor cDNA could be accomplished using L-NASPA and the oocyte system, although this is an arduous route. However, an immediate use of this assay is to test orphan G protein-coupled receptor DNAs, such as mouse EDG-2, which has been proposed to encode an LPA receptor (Hecht *et al.*, 1996). EDG-2 is represented many times in the Genbank database of Expressed Sequence Tags (dbEST). We

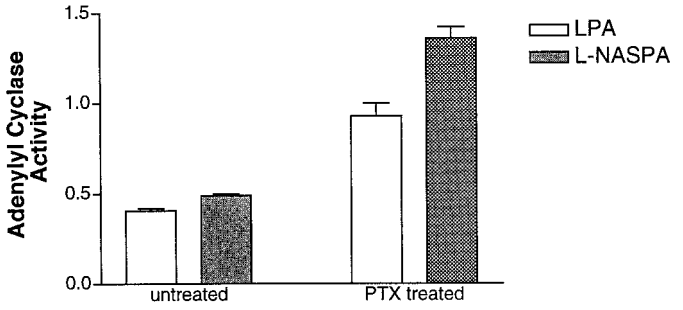

Fig. 4. Inhibition of adenylyl cyclase by L-NASPA and LPA is pertussis-toxin sensitive. Cells treated with 100 ng/ml pertussis toxin overnight do not show inhibition of adenylyl cyclase activity on addition of 1 μM L-NASPA or 1 μM LPA, whereas untreated cells show significant inhibition. Only the highest dose tested is shown; each value was determined in triplicate.

obtained one of the underlying mouse cDNAs (clone ID# 515216) from the I.M.A.G.E. Consortium (Lennon and Auffray, 1996). After subcloning the full translational open reading frame into the expression plasmid, pcDNA-3, we transcribed mRNA *in vitro* in the presence of a guanine nucleotide capping analog, injected this mRNA into oocytes and recorded responses to L-NASPA after 4 days. Although responses to LPA and to control compounds in parallel oocytes (e.g., carbachol applied to oocytes injected with M_1 muscarinic acetylcholine receptor mRNA) were robust (not shown), no response was observed with EDG-2 mRNA injected oocytes when L-NASPA was applied at concentrations up to 10 μM (Fig. 5C).

Discussion

The goal of this study was to identify a low affinity blocker of LPA mediated responses in mammalian cells. We found instead that in human breast carcinoma MDA MB231 cells, L-NASPA acts as a high potency LPA mimetic, and as expected of a receptor-mediated event, the D- stereoisomer was distinctly less potent. Our contention that NASPA acts at an LPA receptor is argued both by its obvious structural similarity to the ethanolamine-based phosphoric acid [which is a full LPA mimetic (Lynch *et al.*, 1997)] and to LPA (Fig. 1) and by the ability of LPA to de-sensitize cells to subsequent NASPA application (Fig. 3). That NASPA is subtype-selective is shown by its antagonism of an amphibian LPA response (Liliom *et al.*, 1996a) and suggested by the unidirectional cross-desensitization with LPA.

Several aspects of our findings are significant. First, we have extended the structure-activity relationship profile of lipid phosphoric acid mediators. Second, to our knowledge, ours is the first demonstration of stereoselectivity by an LPA receptor. Third, the mammalian selectivity of NASPA's activity revives the potential of the oocyte as an expression cloning tool. Finally, we provide additional evidence for multiple LPA receptor subtypes.

Our previous study of the structure/activity relationship of LPA suggested the importance of the distance between the phosphate head group and a dissociable proton (five bond lengths) (Lynch *et al.*, 1997). This distance is maintained in the ethanolamine and serine-based compounds (Fig. 1); it is the addition of the carboxylate moiety to the second carbon of ethanolamine that confers selectivity, whereas potency is retained only when the asymmetric center is in the L-configuration. When the ethanolamine based lipid mediator anandamide (*N*-arachidonyl ethanolamide) is modified by the addition of a methyl group at the equivalent carbon, the resulting (*R*)-methanandamide retained potency as a cannabimimetic but was resistant to degradation by amidase (Pertwee and Makriannis, 1994). Perhaps L-NASPA will also be found to have enhanced metabolic stability.

Although evidence exists for much of the activity of LPA that results from its interaction with receptors, a puzzling finding has been the lack of stereoselectivity of putative LPA receptor ligands. As far as we are aware, D-LPA has not been tested, but both stereoisomers of the ether-containing analog (1-alkyl-LPA) were shown to be equipotent in promoting aggregation of human platelets (Simon *et al.*, 1982) and mobilizing calcium in human A431 cells (Jalink *et al.*, 1995). Sugiura *et al.* (1994) concluded that the LPA receptor lacked a stereospecific requirement because of the ability of lipid phosphoric acids that lack chiral centers to act as agonists. Although Sugiura et al. did not compare D- and L- stereoisomers of NASPA for their ability to antagonize the LPA response in platelets, L- and D-NASPA were found to be equipotent when blocking LPA-induced chloride conductances in frog oocytes (Liliom *et al.*, 1996a). Ours, therefore, is the first demonstration of the expectation that an LPA receptor would discriminate between ligand stereoisomers.

An obvious practical application of our finding is the ready assaying of putative (i.e., ligand unknown of "orphan") LPA receptor DNAs in the frog oocyte. Two claims are now published for the cloning of an LPA receptor DNA. One DNA clone, termed PSP24, was from *X. laevis* oocytes (Guo *et al.*, 1996), so by definition, L-NASPA is not an agonist at this receptor [rather, L(D)-NASPA is an antagonist of the oocyte LPA response]. The second DNA clone is the mammalian orphan G protein-coupled receptor EDG-2 that was renamed *vzg*-1 by Hecht *et al.* (1996). We obtained and tested mouse EDG-2/*vzg*-1, and because we found that it did not confer L-NASPA responsiveness on the oocyte, we conclude that the EDG-2/*vzg*-1 protein is not the NASPA-preferring subtype of LPA receptor. However, we feel compelled to echo the contention of Moolenaar *et al.* (1997) that until and unless these putative LPA receptor DNAs are shown to confer stimulus-response in an LPA-naive cell *and* to bind LPA, claims of LPA receptor cloning are tentative.

The simplest explanation for our findings is that at least two LPA receptor subtypes exist, both of which are equally accessible to LPA and the ethanolamine-based phosphoric acid compound. L-NASPA is able to interact with only one of the subtypes at the dose range tested, and this subtype discriminates further in that it prefers L-NASPA over D-NASPA. The subtypes may also be coupled to different G proteins with different efficiencies. LPA is thought to mediate calcium mobilization through a G_q coupling,and inhibit adenylyl cyclase activation through a G_i coupling. The dose-response curves in Fig. 2 show that neither D- nor L-NASPA is a full agonist with respect to calcium mobilization, whereas both are fully effective in inhibiting adenylyl cyclase activation. This may be explained by a tighter coupling of the NASPA-specific receptor with the G_i proteins. An alternate explanation is that because L-NASPA is a partial agonist (calcium response), there might be present sufficient spare receptors such that L-NASPA would desensitize its own re-

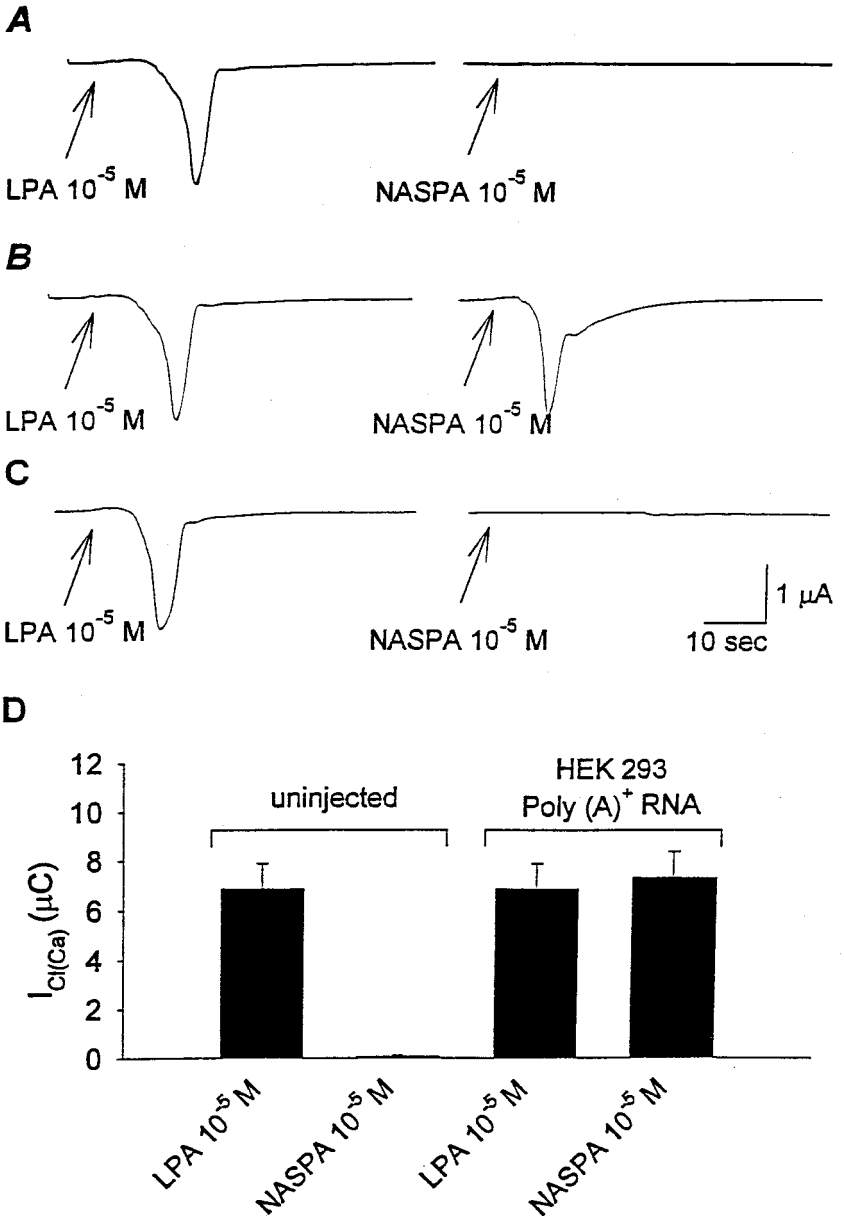

Fig. 5. Response of frog oocytes to applied L-NASPA. A, At an applied concentration of 10 μM (30 μl), LPA, but not L-NASPA, induces inward currents in uninjected oocytes (n = 5). The average of responses was 6.85 ± 1.03 μC. The current developed after a delay of 5–15 sec and consisted of a fast inward component followed by a fluctuating relaxation over several seconds. *Traces,* typical for $I_{Cl(Ca)}$ induced by G protein-coupled receptors in *X. laevis* oocytes. B, HEK 293 poly(A)$^+$ RNA injected oocytes developed inward currents to application of 10 μM L-NASPA or LPA. The average responses were 7.3 ± 1.06 μC (n = 7) and 6.7 ± 0.98 μC (n = 5) for L-NASPA and LPA, respectively. There was no statistically significant difference in the mean of the three groups. C, LPA but not L-NASPA (30 μl, 10 μM) induce $I_{Cl(Ca)}$ in oocytes injected with mouse EDG-2 mRNA. $I_{Cl(Ca)}$ induced by LPA were not different compared with the LPA responses in uninjected or HEK 293 poly (A)$^+$ RNA injected oocytes. The average LPA induced current was 6.95 ± 1.1 μC (n = 5). $I_{Cl(Ca)}$ induced by NASPA (10 μM) was 0.8 μC (n = 6).

sponse, but would not fully desensitize the response to subsequent LPA application. Thus our results could be obtained with a single receptor if the amount of spare receptors and degree of partial agonism were sufficient.

Liliom et al. (1996b) have proposed multiple receptor subtypes for LPA-related compounds based on unidirectional desensitization by 2,3-cyclo-LPA and LPA in oocytes. However, L-NASPA was found to block both the 2,3-cyclo-LPA and the LPA responses in oocytes (Liliom *et al.*, 1996a). From present information, it is difficult to distinguish between the possibilities of three separate receptors versus two receptors wherein the amphibian and mammalian receptors have markedly different ligand selectivities. The possibility of orthologous receptors exhibiting markedly different ligand specificities is supported by the existence of an angiotensin II AT_1 ligand, Sar1Ile8-angII, which is an agonist at the *X. laevis* AT_1 receptor but is a competitive antagonist at the orthologous mammalian receptor (Ji *et al.*, 1993).

In conclusion, our study is significant because it has identified a subtype-selective LPA agonist with high potency and stereospecificity. Future work will further define the structural requirements for receptor interaction, including the addition of other functionalities to the second carbon of the ethanolamine backbone. Finally, the application of L-NASPA to frog oocytes might enable the identification of orphan G protein-coupled receptors as potential LPA receptors.

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