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Identification and Characterization of a Lysophosphatidic Acid Receptor

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SUMMARY

A specific binding site for 1-[^{3}H]stearoyl-lysophosphatidic acid (stearoyl-LPA) was identified and characterized in membranes prepared from rat brain and Swiss 3T3 fibroblasts. Specific binding of [^{3}H]LPA to these sites was protein dependent, was saturable, reached equilibrium in 15 min, and was displacable by the addition of excess unlabeled LPA. Scatchard analysis of saturation binding experiments indicated that these sites had affinities of 2.0 ± 0.5 nM and 5.4 ± 2.6 nM and densities of 19 ± 3 fmol/μg of protein and 38 ± 6 fmol/μg of protein in rat brain and 3T3 cell membranes, respectively. Various LPAs, with different acyl groups in the *sn*-1-position, competed with [^{3}H]LPA for these binding sites, with a rank order of potency of 1-oleoyl-LPA > 1-stearoyl-LPA = 1-palmitoyl-LPA > 1-myristoyl-LPA. Phosphatidic acid also bound to these sites, but with lower affinity than any LPA tested. Neither lysophosphatidylcholine, lysophosphatidylethanolamine, nor any free fatty acid competed with [^{3}H]LPA for these binding sites. Binding of [^{3}H]LPA to these sites was regulated by nonhydrolyzable guanine nucleotides in both rat brain and 3T3 cell membranes. Furthermore, in 3T3 cells, these sites were regulated by cell density. It was subsequently determined that LPA induced a transient increase in intracellular Ca^{2+} levels in 3T3 cells. The concentrations required for this response, as well as the rank order of potency of the various LPAs and phosphatidic acid, correlated with the affinity of these compounds for the [^{3}H]LPA binding site. These results suggest that the specific, high affinity, binding sites for [^{3}H]LPA are G protein-coupled receptors.

LPA (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate) is a naturally occurring lipid that is capable of inducing a multiplicity of biological effects. Exogenously added LPA has been shown to promote platelet aggregation (1, 2), alter neuronal cell morphology (3), induce smooth muscle contraction (4, 5), elicit a chemotactic response in *Dictyostelium* (6), and trigger DNA synthesis and cell division in a number of different cell types (7, 8). Additionally, LPA can regulate phospholipid turnover (7, 9, 10), mobilize Ca^{2+} (8), inhibit adenylate cyclase (11, 12), and activate *ras*-type G proteins (13, 14). It was also shown that some LPA-induced responses were modulated by guanine nucleotides and inhibited by pertussis toxin (7, 9, 12, 15), leading to speculation that LPA interacts with specific G protein-coupled receptors.

As a first step in the identification and characterization of an LPA receptor, [^{3}H]LPA ligand binding assays were performed. Specific, high affinity, binding sites were found in membranes prepared from rat tissues and 3T3 cells. When intracellular Ca^{2+} levels in 3T3 cells were monitored by fura-2 fluorescence, the potencies of various LPAs in this response correlated with their affinities for the [^{3}H]LPA binding sites, a result consistent with these binding sites being LPA receptors.

Experimental Procedures

Materials. [^{3}H]Stearoyl-LPA (1-[^{3}H]stearoyl-*sn*-glycerol-3-phosphate) (specific activity, 180 Ci/mmol) was obtained from DuPont-New England Nuclear (Milford, MA). Nonradioactive lipids, including 1,2-dipalmitoylphosphatidic acid, oleoyl-LPC (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine), oleoyl-LPE (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), stearoyl-LPC (1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine), stearoyl-LPE (1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), lyso-PAF (1-oleoyl-2-acetoyl-*sn*-glycero-3-phosphocholine), PAF (1-alkyl-2-acetoyl-*sn*-glycero-3-phosphocholine), oleoyl-LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate), stearoyl-LPA (1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphate), myristoyl-LPA (1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphate), and palmitoyl-LPA (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate), were purchased from Avanti Polar Lipids (Alabaster, AL) and were >98% pure, as determined by thin layer chromatography. Oleic, stearic, palmitic, and myristic acids were obtained from Aldrich Chemical Co. (St. Louis, MO). GTPγS tetralithium salt, Gpp(NH)p tetralithium salt, and other nucleotides were obtained from Boehringer Mannheim (Indianapolis, IN). Fura-2/acetoxymethyl ester was purchased from Molecular Probes Inc. (Eugene, OR).

Cell culture. Swiss 3T3 cells were obtained from the American

ABBREVIATIONS: LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; Gpp(NH)p, guanosine-5′-(β,γ-imido)diphosphate; PAF, platelet-activating factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATPγS, adenosine-5′-*O*-(3-thio)triphosphate; HBSS, Hanks' balanced salt solution.

Type Culture Collection (Bethesda, MD) and were used within 20 passages of receipt. The cells were grown in Dulbecco's modified Eagle's medium (GIBCO) with 4.5 g/liter glucose, supplemented with 10% calf serum (HyClone, Logan, UT), in a humidified atmosphere of 95% air/5% CO_2 at 37°. Cells were allowed to grow to no greater than 60% confluency, unless otherwise indicated, and were then either harvested for binding studies or plated onto 10-cm diameter Petri dishes for Ca^{2+} mobilization experiments.

Membrane preparation. Male Sprague-Dawley rats (approximately 250 g) were euthanized using CO_2 and then decapitated. The indicated organs were removed and placed in 10 ml of ice-cold 20 mM Tris·HCl, pH 7.5 (assay buffer). Because the [^{3}H]LPA binding site appeared to be unaffected by freezing (data not shown), whole frozen rat brains were also obtained from Pel-Freez (Rogers, AR). Frozen brains were defrosted at room temperature in assay buffer (1 brain/10 ml) before use. For binding studies performed with 3T3 fibroblast membranes, cells were mechanically removed from the culture dish and resuspended in assay buffer (10%, v/v).

Tissues and cells were disrupted using a Polytron homogenizer. Large pieces of cell debris were removed by centrifugation of the homogenate at 1000 × *g* for 15 min at 4°. A particulate fraction was isolated from the resulting supernatant by centrifugation (40,000 × *g* for 15 min at 4°). This high-speed pellet was resuspended in assay buffer to a final protein concentration of 4 mg/ml and is referred to as the membrane preparation. Membrane preparations either were used immediately or were stored frozen at −70° until required. Frozen membrane preparations retained, for at least 4 weeks, 100% of the specific [^{3}H]LPA binding measured in fresh membrane preparations.

Assessment of [^{3}H]LPA metabolism. To determine whether the [^{3}H]LPA in the binding experiments had been converted to either free fatty acid and/or phosphatidic acid by the action of lysophospholipases and acyltransferases, membranes (80 μg/ml) were incubated with [^{3}H]LPA (0.5 nM) in assay buffer for 30 min at 37°. The radioactive lipids were extracted from the membranes by the method of Bligh and Dyer (16) and were separated by thin layer chromatography on silica gel G plates (Analtech, Wilmington, DE), using a solvent system consisting of chloroform/methanol/acetic acid/water (100:60:16:8, v/v).

Binding assays. Binding assays were performed by incubating membranes (20 μg/ml) with [^{3}H]LPA (2.5 nM, approximately 396,000 dpm), 0.5 mM $CuSO_4$, and 20 mM Tris, pH 7.5, in an assay volume of 0.5 ml. Reactions were initiated by the addition of ligand and were incubated for 30 min at 30° unless stated otherwise. Bound [^{3}H]LPA was separated from free radioligand by gel filtration, using Sephadex G-25 (PD-10) columns obtained from Pharmacia (Piscataway, NJ). Columns were pre-equilibrated in assay buffer and maintained at 4°. The column eluates were collected, mixed with 8 ml of scintillant (Ready Safe; Beckman, Palo Alto, CA), and then quantified by liquid scintillation counting. Under these assay conditions, <10% of total radioligand was bound to the membranes.

To examine the effect of pH on [^{3}H]LPA binding, assays were carried out as described above but with the appropriate buffer for the pH to be tested, as follows: pH 3.5–5.0, sodium acetate; pH 5.5–6.5, 4-morpholineethanesulfonic acid; pH 7.0–7.5, HEPES; pH 7.5–9.0, Tris·HCl; pH 9.5–11.0, 3-(cyclohexylamine)-1-propanesulfonic acid. All buffers were used at a concentration of 20 mM.

Total binding was defined as the amount of radioactivity bound to the membranes in the absence of competing ligand. Nonspecific binding was defined as the amount of [^{3}H]LPA binding that occurred in the presence of a 1000-fold excess of nonradioactive stearoyl-LPA. Specific binding was defined as the difference between total and nonspecific binding.

Fluorescence measurements of cellular Ca^{2+} levels. Intracellular Ca^{2+} levels were measured by fluorescence, using fura-2. Cells were grown for 24 hr in growth medium and then incubated for an additional 24 hr in Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum. The cells were then incubated with fura-2/acetoxymethyl ester (1 μg/ml) for 30 min. The cell monolayer was washed with HBSS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.5 (GIBCO), and was incubated in HBSS for an additional 30 min. The cells were scraped off the dish, centrifuged (1000 × *g* for 3 min), and resuspended in HBSS (10^6 cells/ml). For Ca^{2+} fluorescence measurements, 2 ml of cell suspension were transferred to quartz cuvettes, maintained at 37°, and constantly stirred throughout the duration of the experiment. Calcium concentrations were calculated by the method of Grynkiewicz *et al.* (17), using a Perkin-Elmer LS 50B fluorimeter. For calibration, maximum Ca^{2+} levels were obtained by lysis of the cells with Triton X-100 and minimum levels were measured by the subsequent addition of EDTA. Aliquots of cells were treated with a single dose of LPA. Data were calculated as the ratio of the maximal Ca^{2+} response observed.

Results

Development of the [^{3}H]stearoyl-LPA binding assay. Initial experiments were performed using rat brain membranes because of the availability of large amounts of tissue and because these membranes had previously been shown, by photocross-linking techniques, to contain a putative LPA-binding protein (18). To determine whether the radioligand was enzymatically degraded, membranes were incubated with [^{3}H]LPA for 30 min, the lipids were extracted and separated by thin layer chromatography, and the amount of radioactivity associated with phosphatidic acid, LPA, and free fatty acids was determined. More than 70% of the [^{3}H]LPA was converted into free fatty acid (Table 1), suggesting that most of the ligand had been degraded by a lysophospholipase. It has been previously reported that many lysophospholipases are inhibited by metal ions (19, 20). Therefore, a variety of metal ions were tested for their ability to inhibit ligand degradation and thus maximize the specific binding signal. It was found that addition of 0.5 mM $CuSO_4$ to the assay buffer inhibited degradation of [^{3}H]LPA (Table 1), and $CuSO_4$ was, therefore, used in all subsequent experiments.

The binding of [^{3}H]LPA to rat brain membranes was protein concentration dependent and abolished in boiled membranes (data not shown). The pH optimum for [^{3}H]LPA binding to rat brain membranes was 7–7.5. When 20 μg/ml membranes were used, total binding was approximately 19,000 cpm, of which approximately 7,500 cpm was specific binding. Cations, including $MgCl_2$, $CaCl_2$, NaCl, and KCl (10 mM), had no effect on the specific binding of [^{3}H]LPA to rat brain membranes (data not shown).

Tissue distribution of [^{3}H]LPA binding sites. To determine the distribution of [^{3}H]LPA binding sites in rat tissues, assays were performed using membranes prepared from a variety of tissues. Significant amounts of specific binding of [^{3}H]

TABLE 1

Recovery of radiolabeled ligand after incubation with rat brain membranes

To determine the stability of [^{3}H]LPA in the binding assay, membranes (80 μg/ml) were incubated with the ligand (2.5 nM) for 30 min at 37°. The lipids were extracted with organic solvent and >95% of the radioactivity was recovered. The extracted lipids were separated by thin layer chromatography and the amount of radioactivity found to chromatograph with authentic standards was expressed as a percentage of the total recovered. Data are shown as means for three experiments.

Treatment	Radioactivity		
	LPA	Phosphatidic acid	Fatty acid
	% of total		
None	20	2	78
$CuSO_4$	98	0	2

LPA to membranes were detected in all rat tissues tested (Table 2). The greatest amount of specific binding was detected in rat brain membranes, followed by spleen, liver, and lung, with most other tissues containing a moderate number of binding sites. Relatively small amounts of specific binding were detected in skeletal muscle.

Association and dissociation of [^{3}H]LPA in rat brain membranes. The association of [^{3}H]LPA with specific binding sites on rat brain membranes was time dependent, reached equilibrium by 15 min, and remained constant for at least 1 hr (Fig. 1). Reversibility of [^{3}H]LPA binding was demonstrated by first incubating the membranes with [^{3}H]LPA for 30 min and then adding a vast excess of nonradiolabeled LPA to the reaction mixture. Greater than 50% of bound [^{3}H]LPA was displaced from specific binding sites in <1 min and all of the ligand was displaced in 5 min (Fig. 1).

Scatchard analysis of [^{3}H]LPA binding. Saturation binding experiments were performed with [^{3}H]LPA (0.1–10 nM), and the data were transformed by Scatchard analysis (Fig. 2). Rat brain membranes were found to contain a single class of high affinity (2.0 ± 0.5 nM) binding sites, at a density of 19 ± 3 fmol/μg of protein.

Specificity of [^{3}H]LPA binding. Competition binding experiments were performed using rat brain membranes and a variety of LPAs with different fatty acids in the *sn*-1-position (Fig. 3). The rank order of potency of the LPAs for the [^{3}H]LPA binding site was oleoyl-LPA > stearoyl-LPA = palmitoyl-LPA > myristoyl-LPA. Phosphatidic acid was 100-fold less potent than myristoyl-LPA. The free fatty acids oleic, stearic, palmitic, and myristic acids, as well as other lipids such as PAF, lyso-PAF (at 10 μM), stearoyl-LPC, stearoyl-LPE, oleoyl-LPC, and oleoyl-LPE (at 100 μM), were unable to compete for [^{3}H]LPA binding sites (data not shown).

Characterization of [^{3}H]LPA binding to 3T3 cell membranes. To determine whether the [^{3}H]LPA binding sites were receptors, it was necessary to identify a system in which a

TABLE 2

Distribution of [^{3}H]LPA binding sites in rat tissues

Membranes (80 μg/ml) were prepared from rat tissues and incubated in the presence of 2.5 nM [^{3}H]LPA only (total binding) or in the additional presence of 2.5 μM nonradioactive LPA (nonspecific binding). Data are shown as means ± standard errors for six determinations.

Tissue	Specific binding
	fmol/μg of protein
Brain	6.86 ± 0.23
Heart	0.90 ± 0.07
Lung	1.23 ± 0.11
Testes	1.34 ± 0.01
Spleen	1.61 ± 0.30
Liver	1.67 ± 0.01
Kidney	0.85 ± 0.03
Stomach	0.60 ± 0.01
Large intestine	0.38 ± 0.03
Ileum	0.49 ± 0.01
Pancreas	0.84 ± 0.02
Bladder	0.88 ± 0.06
Skeletal muscle	0.18 ± 0.01

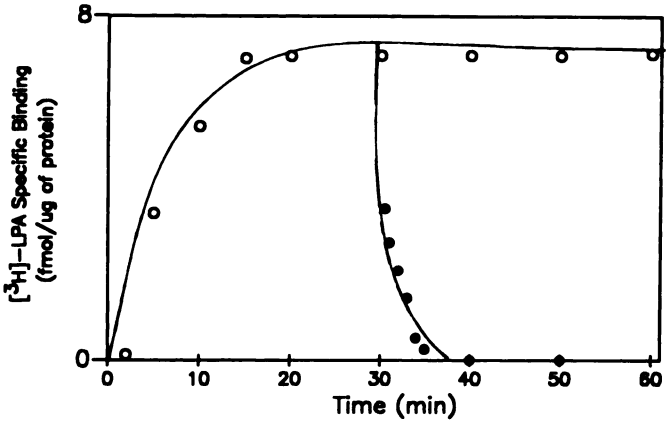

Fig. 1. Association and dissociation of [^{3}H]LPA binding to rat brain membranes. The time course of [^{3}H]LPA association (○) to specific sites in rat brain membranes was determined by incubating 20 μg/ml membranes with 2.5 nM [^{3}H]LPA alone (total binding) or in the additional presence of 2.5 μM nonradiolabeled LPA (nonspecific binding) for the indicated times. The time course of dissociation (●) was determined by incubating rat brain membranes with [^{3}H]LPA for 30 min, to obtain equilibrium conditions, and then adding unlabeled LPA (2.5 μM) and incubating membranes for various times thereafter. Each *point* represents the mean specific binding for three determinations, with a standard error of <10%.

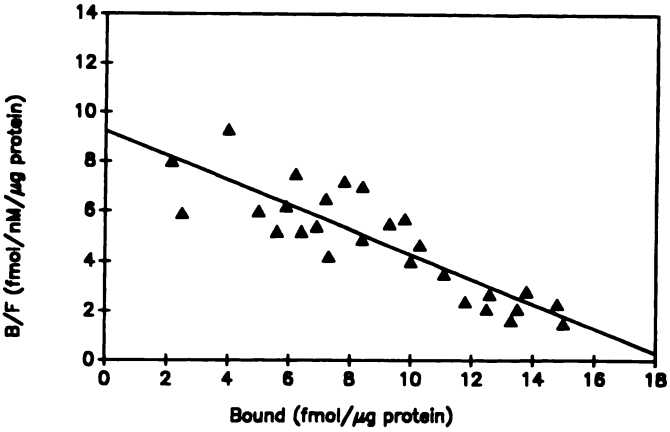

Fig. 2. Scatchard analysis of [^{3}H]LPA binding to rat brain membranes. Rat brain membranes (20 μg/ml) were incubated with 0.1–10 nM [^{3}H]LPA alone (total binding) or in the additional presence of 10 μM unlabeled LPA (nonspecific binding). Each *point* represents the mean specific binding for three determinations. The data shown are from three separate experiments.

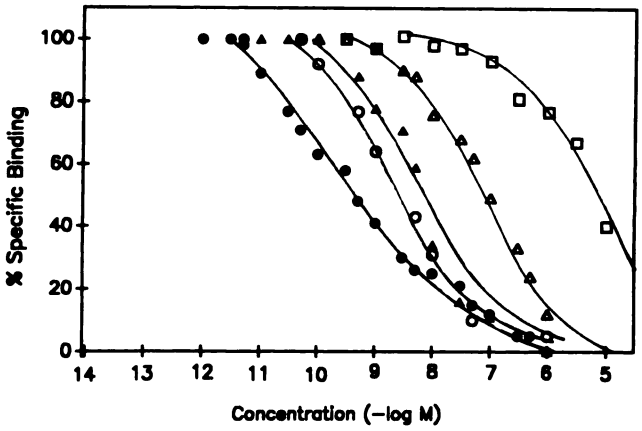

Fig. 3. Competition with [^{3}H]LPA binding to rat brain membranes by LPAs and phosphatidic acid. Rat brain membranes (20 μg/ml) were incubated with [^{3}H]LPA alone or in the additional presence of various concentrations of unlabeled competing ligand. ○, 1-Stearoyl-LPA; ●, 1-oleoyl-LPA; △, 1-myristoyl-LPA; ▲, 1-palmitoyl-LPA; □, phosphatidic acid. Each *point* represents the mean ± standard error for four determinations. Standard errors were <10% in each case.

response to LPA could be readily quantified and correlated with the binding data. Because it had been previously reported that 3T3 cells responded to LPA by increasing the levels of intracellular Ca^{2+} (8), membranes from these cells were examined for specific [^{3}H]LPA binding sites. Saturation binding experiments demonstrated the presence of a single class of high affinity (5.4 ± 2.6 nM) binding sites, with a density of 38 ± 6 fmol/μg of protein, in 3T3 cell membranes (Fig. 4A). Furthermore, competition binding experiments indicated that the rank orders of potency and affinity of the various LPAs tested (Fig. 4B) were essentially the same as had been observed in rat brain (Figs. 2 and 3).

Effects of nucleotide analogues on [^{3}H]LPA binding to specific sites. Cellular responses to LPA are reported to be modulated by guanine nucleotides, suggesting that LPA receptors are G protein coupled (7, 9, 12, 15). Experiments were performed to determine the effects of guanine nucleotides on [^{3}H]LPA binding to rat brain and to 3T3 cell membranes. However, because divalent cations reduce the rate of guanine nucleotide exchange (21), $CuSO_4$ had to be eliminated from the binding assay. It was found that four freeze/thaw cycles destroyed the ligand-degrading lysophospholipase (data not shown) but had no appreciable effect on either the affinity or the density of the [^{3}H]LPA binding sites (Fig. 5A). Therefore, [^{3}H]LPA binding experiments were performed using frozen/thawed membranes in the absence of $CuSO_4$. Both GTPγS and Gpp(NH)p induced a dose-dependent decrease in specific [^{3}H]LPA binding to 3T3 cell membranes (Fig. 5B). However, neither GDP, ATP, ATPγS, nor CTP reduced specific binding. Scatchard analysis indicated that GTPγS treatment decreased the affinity of this binding site from 5.4 ± 0.6 to 2.1 ± 0.8 nM (means ± standard deviations from three experiments). The density of the [^{3}H]LPA binding sites on membranes from 3T3 cells was unaffected by this treatment (Fig. 5A). Similar data were obtained using rat brain membranes (data not shown). Taken together, these data indicate that the [^{3}H]LPA binding site is regulated by guanine nucleotides, and they suggest that this site may be a G protein-coupled receptor.

Regulation of [^{3}H]LPA binding sites by cell density. LPA is a mitogen in 3T3 cells (22), a cell line whose growth is arrested when cells reach confluency (23). Therefore, we pos-

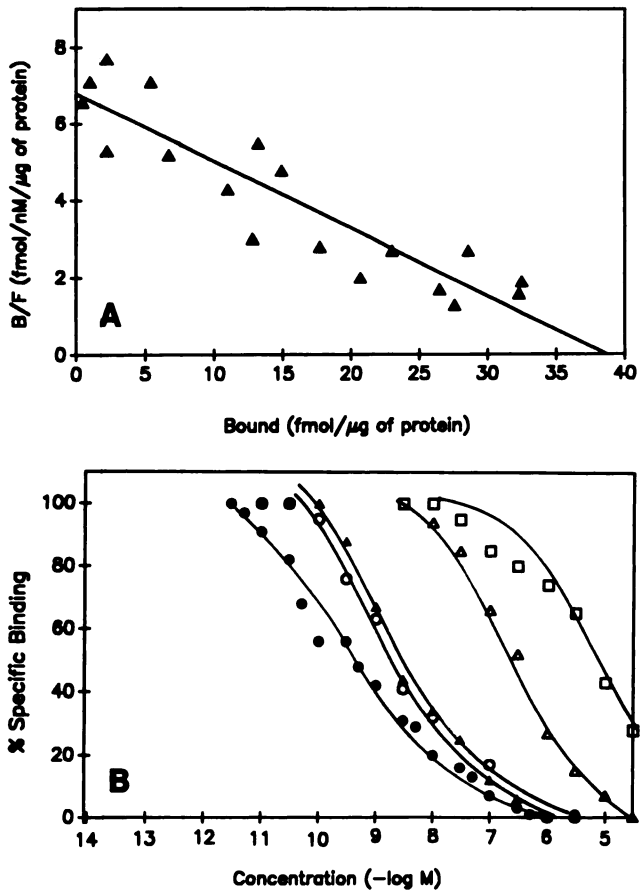

Fig. 4. Characterization of [^{3}H]LPA binding to 3T3 cell membranes. A, Scatchard analysis of [^{3}H]LPA binding to 3T3 cell membranes. Membranes (20 μg/ml) were incubated with 0.1–10 nM [^{3}H]LPA only (total binding) or in the additional presence of 10 μM nonradioactive LPA (nonspecific binding). B, Competition with [^{3}H]LPA binding to 3T3 cell membranes. Membranes (20 μg/ml) were incubated with [^{3}H]LPA only or in the additional presence of various concentrations of unlabeled competing ligand. ○, 1-Stearoyl-LPA; ●, 1-oleoyl-LPA; △, 1-myristoyl-LPA; ▲, 1-palmitoyl-LPA; □, phosphatidic acid. Each *point* represents the mean ± standard error of three experiments. Standard errors were <10% in each case.

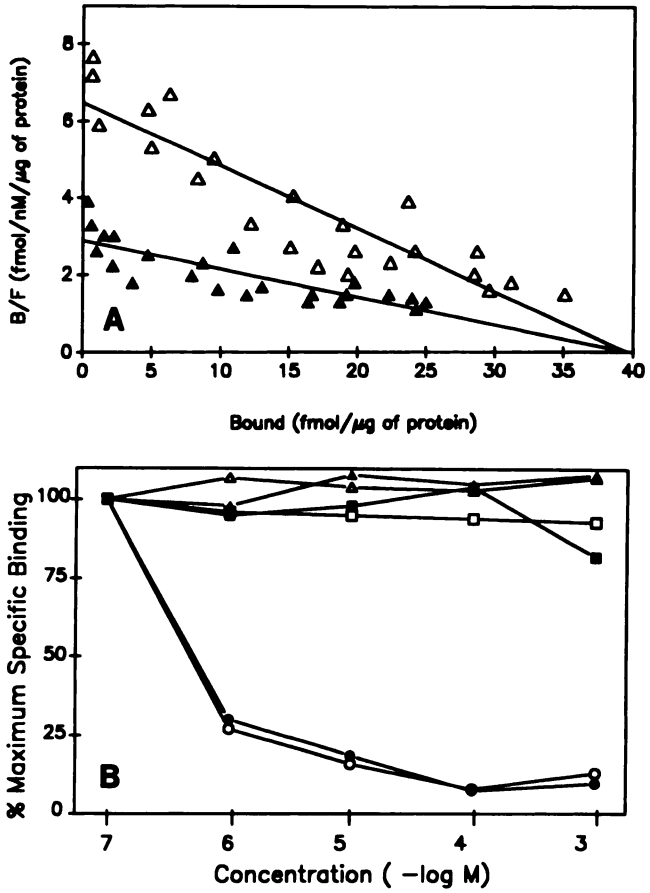

Fig. 5. Effects of nucleotides on [^{3}H]LPA binding to 3T3 cell membranes. A, Scatchard analysis of [^{3}H]LPA binding to 3T3 cell membranes. Saturation binding experiments were performed as described for Fig. 4 but in the absence of $CuSO_4$, using membranes that had been frozen and thawed. Assays were performed either in the absence (△) or in the presence (▲) of 10 μM GTPγS. Each *point* represents the mean specific binding for three determinations. The data shown are from three separate experiments. B, Effects of nucleotides on [^{3}H]LPA binding. Nucleotides were incubated with 3T3 cell membranes, and the effects on specific binding are shown. ○, GTPγS; ●, Gpp(NH)p; ▲, ATPγS; △, ATP; □, CTP; ■, GDP. These data represent the means from three separate experiments (standard deviation was <15%).

tulated that the LPA receptor may be down-regulated in confluent cells. If the [^{3}H]LPA binding site that we identified in these studies is a receptor, then it would be similarly regulated. Cells were grown to various densities and harvested, and the resulting membranes were examined in [^{3}H]LPA binding experiments. Cells grown to either 30% confluency (17×10^3 cells/cm^2) or 60% confluency (17×10^3 cells/cm^2) had substantial and similar amounts of [^{3}H]LPA binding. In contrast, confluent cells (57×10^3 cells/cm) had at least a 5-fold reduction in [^{3}H]LPA binding, compared with subconfluent cells (Table 3).

LPA mobilization of Ca^{2+} in 3T3 cells. A correlation between the rank order of potencies of agonists and antagonists for a biological response and their affinities in binding studies provides important evidence that a binding site is a receptor. Because LPA induces a transient increase in intracellular Ca^{2+} levels in fibroblasts (8), this response was examined using fura-2-loaded 3T3 cells. Treatment of 3T3 cells with all LPAs tested evoked a concentration-dependent increase in intracellular Ca^{2+} levels (Fig. 6). The maximal increase in intracellular Ca^{2+} levels was about 2.5 times that measured before treatment. However, the dose required to induce this response was different for each LPA tested. Phosphatidic acid was unable to induce a significant increase in 3T3 cell Ca^{2+} fluorescence at concentrations below 3 μM. Higher concentrations were found to cause cell lysis.

The potency and efficacy of each LPA in these experiments were similar to those previously reported using fibroblasts (8). The dose of each LPA that induced a half-maximal increase in intracellular Ca^{2+} levels (ED_{50}) was calculated (Table 4) and compared with the affinity of the LPA for the [^{3}H]LPA binding site (Table 4), and a high degree of correlation was observed.

TABLE 3

Cell density regulation of [^{3}H]LPA binding to 3T3 cell membranes

Membranes were prepared from 3T3 fibroblasts grown to the indicated cell densities, which corresponded to 30, 70, and 100% confluency. Membranes (20 μg/ml, final concentration) were incubated in an assay mixture containing [^{3}H]LPA (2.5 nM) and $CuSO_4$ (500 μM) in 20 mM Tris·HCl, pH 7.5. Data are shown as means ± standard errors from four experiments.

Cell density	Specific [^{3}H]LPA binding
cells/cm^2	*fmol/μg of protein*
17×10^3	5.32 ± 0.26
34×10^3	4.69 ± 0.27
57×10^3	0.85 ± 0.14

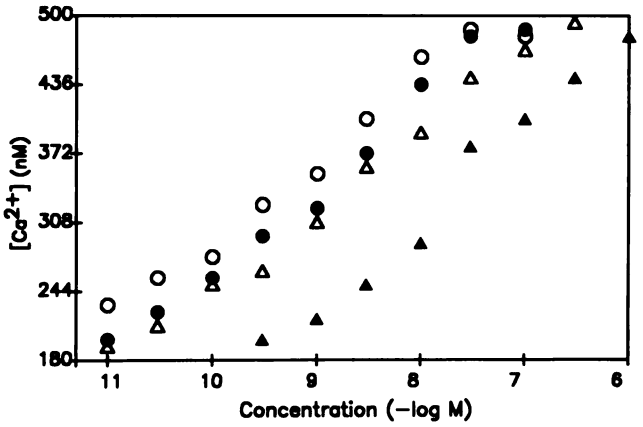

Fig. 6. Dose-response curve for the effect of LPAs with different acyl chains on intracellular Ca^{2+} levels in 3T3 cells. Fura-2-labeled 3T3 cells were treated with increasing concentrations of various LPAs [○, 1-stearoyl-LPA; ●, 1-oleoyl-LPA; △, 1-myristoyl-LPA; ▲, 1-palmitoyl-LPA] and the peak increase in intracellular Ca^{2+} levels was measured fluorimetrically. The data shown are from a representitive experiment.

Discussion

Many diverse biologically active lipids exist, some of which interact with specific membrane-associated receptors. Although LPA is biologically active, receptors for LPA have not been characterized; however, numerous investigators have hypothesized their existence (24–27). Previous attempts to identify an LPA receptor using [^{32}P]LPA were compromised by high levels of nonspecific and nondisplaceable binding of the ligand (17, 28). Initial experiments in our laboratory showed that [^{3}H]LPA was rapidly degraded to free fatty acid when incubated with membrane preparations, suggesting hydrolysis by a lysophospholipase (Table 1). Therefore, it was essential to develop assay conditions in which the ligand remained intact. Two different methods for circumventing this problem were identified. The first was based on published data that indicated that lysophospholipases are inhibited by a variety of metal ions (19, 20), and $CuSO_4$ was found to be effective in this regard. In the second method, repetitive cycles of freezing and thawing of the membranes also inactivated endogenous lysophospholipases. Neither method appeared to have an appreciable effect on the levels of specific binding observed.

Using conditions that prevented ligand degradation, a specific [^{3}H]LPA binding site was identified and characterized in rat brain and 3T3 cell membranes. The characteristics of [^{3}H]LPA binding to this site (i.e., affinity, capacity, saturability, and specificity) were consistent with those of a receptor. Guanine nucleotides, which regulate cellular responses to LPA (7, 9), also regulate [^{3}H]LPA binding, suggesting that this site is a G protein-coupled receptor. The most convincing evidence for this binding site being a receptor was the demonstration that the rank order of potencies of various LPAs and phosphatidic acid in the binding assays was the same as that found for regulation of intracellular Ca^{2+} levels in 3T3 fibroblasts.

Specific binding sites for [^{3}H]LPA were present in all tissues examined, an observation suggesting that LPA receptors are widely distributed. These results contrast, in part, with data obtained using a photoaffinity analogue of LPA, which labeled an LPA-binding protein in brain but not liver (18). In this study, brain membranes had at least 4 times more specific [^{3}H]LPA binding sites than did liver and, therefore, it is possible

TABLE 4

Correlation between the binding affinity and calcium response in 3T3 cells

The Ca^{2+} response to increasing concentrations of LPAs with different fatty acid chains at the *sn*-1-position was measured in fura-2-loaded 3T3 cells in fluorescence experiments. The concentration of LPA that could induce 50% of the maximal response (ED_{50}) was then determined (means ± standard deviations for four separate experiments). These data were compared with the affinities of the various LPAs in the binding assay (the data shown are the means of three experiments; the standard deviation was approximately 10–12%).

LPA	Binding affinity, IC_{50}	Calcium response, ED_{50}
	nM	*nM*
1-Oleoyl-LPA	0.3	0.9 ± 0.08
1-Stearoyl-LPA	3.0	2.6 ± 0.7
1-Palmitoyl-LPA	6.0	3.1 ± 0.8
1-Myristoyl-LPA	100	19.8 ± 4.6

that this apparent discrepancy is the result of a sensitivity difference between these two assays.

The rank order of potencies of the LPAs in Rat-1 fibroblast mitogenesis assays (7, 8, 22) was the same as we have found in binding assays performed on membranes isolated from 3T3 fibroblasts. However, the proliferative response required micromolar concentrations of LPA, in distinct contrast to the Ca^{2+} response, for which nanomolar concentrations of LPA were effective. It may be that the mitogenic response is mediated by a different LPA receptor than described here. Alternatively, because the mitogenic response requires hours to occur, degradation of LPA during this time may reduce the actual amount of LPA present.

LPA is active in organisms as phylogenetically diverse as fungi (6), amphibians (3), and mammals, suggesting that, on the evolutionary scale, this lipid may have emerged early. Significant quantities of LPA are produced after cell activation (28–30) and, when released into the extracellular milieu, may interact with receptors and have either an autocrine or a paracrine effect. Thus, LPA joins the ranks of other biologically active lipids, such as PAF and prostaglandins, that interact with unique membrane-bound receptors. The simplicity of this molecule belies the multiplicity of its effects, and its full biological relevance will not be fully realized until specific antagonists are developed.

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