Lysophosphatidic acid (LPA) receptors are activated differentially by biological fluids: possible role of LPA-binding proteins in activation of LPA receptors

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Abstract **Lysophosphatidic acid (LPA) exerts multiple biological functions through G protein-coupled receptors (EDG2/LPA$_1$, EDG4/LPA$_2$, and EDG7/LPA$_3$) and is present in serum where it is associated with albumin. In this study we examined LPA activity in various biological fluids by measuring the LPA-induced increase in the intracellular concentration of calcium ion in three types of Sf9 insect cells, each expressing one of the LPA receptors. Using this system, we found that EDG2 and EDG4, but not EDG7, were activated strongly by addition of incubated plasma. By contrast, LPA detected in seminal plasma, which contains a low concentration of albumin, selectively activated EDG7. After LPA in these samples was extracted and reconstituted, it activated all three receptors. We also found that serum albumin readily inhibits the activation of EDG7 but not the activation of EDG2 or EDG4. In addition, plasma from Nagase analbuminemic rats but not plasma from control Sprague–Dawley rats was found to strongly activate EDG7, although the plasma of these two types of rats contained equal amounts of LPA and activated both EDG2 and EDG4. The present study shows that serum albumin can negatively regulate EDG7 but not EDG2 or EDG4, and suggests that protein factors are present in seminal plasma and deliver LPA efficiently to EDG7 but not to EDG2 or EDG4. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: EDG7; LPA3; Lysophosphatidic acid; Albumin; Nagase analbuminemic rat; Seminal fluid

1. Introduction

Lysophosphatidic acid (1- or 2-acyl-lysophosphatidic acid; LPA) is a lipid that mediates multiple cellular processes [1–3], including platelet aggregation, smooth muscle contraction, cell proliferation, and cytoskeletal reorganization (e.g. generation of actin stress fibers and inhibition of neurite outgrowth). LPA evokes its multiple effects through G protein-coupled receptors (GPCR) that are specific to LPA. Recent studies have identified a new family of receptor genes for LPA (reviewed in [4,5]). Members of this family include three GPCRs belonging to the EDG (endothelial cell differentiation gene) family, EDG2/LPA1 [6], EDG4/LPA2 [7], and EDG7/LPA3 [8,9]. They appear to have distinct tissue distribution patterns [5–8], indicating specific functions for each receptor. Indeed, it was recently shown by targeted deletion that the EDG2 receptor is required for normal development of inborn and neonatal behavior [10].

LPA has been detected in various biological fluids such as serum [11], and ascitic fluid from ovarian cancer patients [12]. It is also found to a lesser extent in plasma. LPA is also produced by activated platelets [13]. Recent studies on the mechanisms of LPA production have identified several synthetic pathways and identified several phospholipases involved in these pathways [13–15], which may explain why such a variety of LPA species are detected in vivo. Interestingly, each LPA receptor is activated differentially by different LPA species [16], indicating a possible link between each synthetic pathway and each receptor.

In biological fluids, LPA appears to be associated with protein factors. Albumin is an abundant, lipid-binding protein in blood plasma [17], where it occurs at a concentration of around 4 g/dl. The primary role of albumin is to transport fatty acids, but its versatile binding capacity implies additional functions such as transport of drugs and especially, lysophospholipid mediators, including sphingosine 1-phosphate, platelet-activating factor, lysophosphatidylcholine, and LPA. Indeed, it has been demonstrated that serum albumin and other LPA-binding proteins modify the cellular function of LPA [11,18]. This notion strongly indicates that when LPA is detected in biological fluids by lipid analysis using classical methods such as thin-layer chromatography or recently developed mass spectrometry, its presence and activity should also be evaluated in the light of receptor activation. Most mammalian culture cells express one or more LPA receptors [19]. In addition, they also express other receptors for growth factors. Thus, it is difficult to evaluate the presence of LPA in biological fluids such as serum and plasma through activation of LPA receptors, because these biological fluids contain other growth factors that evoke multiple cell responses in the cells. We recently established a bioassay system to evaluate the activation of each of the LPA receptors, individually expressed in Sf9 insect cells by measuring the change in intracellular calcium concentration evoked by the addition of LPA [8,16]. In the absence of LPA receptor expression, these insect cells did not show any calcium response to any of the biological fluids, which enabled us to measure LPA activity in the

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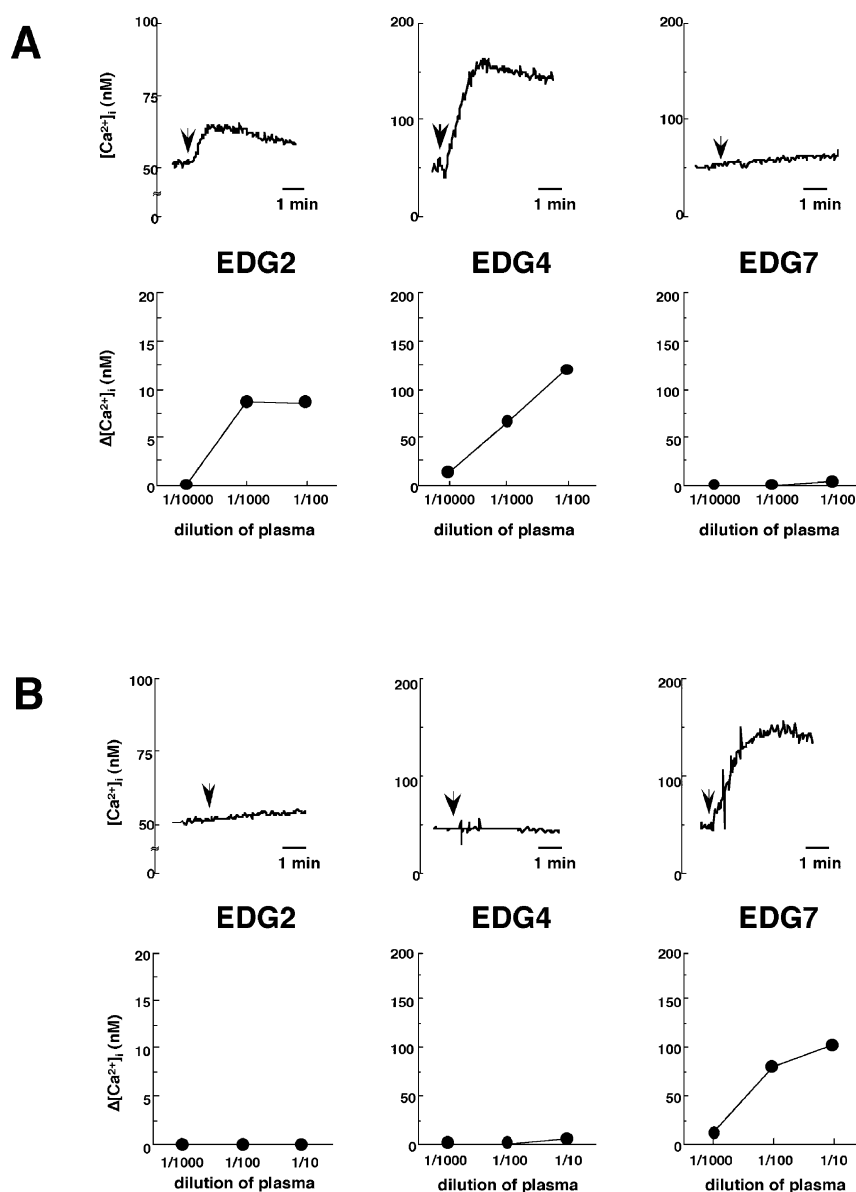E-mail address: jaoki@mol.f.u-tokyo.ac.jp (J. Aoki).

Fig. 1. Differential activation of LPA receptors by incubated blood plasma and seminal plasma. Incubated blood plasma prepared from SDR (A) or freshly prepared seminal plasma from healthy volunteers (B) was added to Fura-2-loaded Sf9 cells expressing human EDG2, EDG4, or EDG7. LPA activities were evaluated by measuring increases in $[Ca^{2+}]_i$ in the insect cells after samples were added to the cells. Arrows indicate the application of the diluted samples (100-fold dilution for LPA extracted from the incubated plasma and 10-fold dilution for LPA extracted from the seminal fluids). Dose–response curves are shown in the lower panels.

biological fluids. In this study, to understand where and how LPA is produced and how it acts on cellular receptors, we measured LPA activity in various biological fluids by evaluating receptor activation using the bioassay system. We demonstrate here that two biological fluids, blood plasma and seminal plasma, differentially activate LPA receptors.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA; fatty acid-free; A-6003) was purchased from Sigma. 1-Oleoyl-LPA (18:1) and 1-[³H]oleoyl-LPA (18:1) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and Amersham Pharmacia Biotech (Buckinghamshire, England), respectively. Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). HPPA (3-(4-hydroxyphenyl) propionic acid) was from Nacalai (Kyoto, Japan).

2.2. Animals

Male Nagase analbuminemic rats (NAR) (8 weeks old) and male Sprague–Dawley rats (SDR) of similar age were purchased from Japan SLC (Shizuoka, Japan). The rats were fed a standard rat chow ad libitum until the time of the study. Albumin in plasma from NAR and SDR was determined using a bromocresol purple dye binding kit from Ono Pharmaceutical (Osaka, Japan).

2.3. Evaluation of LPA receptor activation

LPA receptor activation of biological samples was evaluated by the Ca^{2+} assay using Sf9 cells expressing each LPA receptor as described previously [8,16]. Briefly, agonist-induced Fura-2 AM fluorescence of Fura-2-loaded Sf9 cells expressing the EDG2–4 chimeric receptor, EDG4, or EDG7 in quartz cuvettes was monitored at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm using a CAF-110 spectrofluorometer (Japan Spectroscopy, Tokyo, Japan). As described previously [8], EDG2 expressed in insect Sf9 cells could not mediate Ca^{2+} signaling induced by LPA. We therefore constructed the EDG2–4 chimeric receptor, in which the intracellular
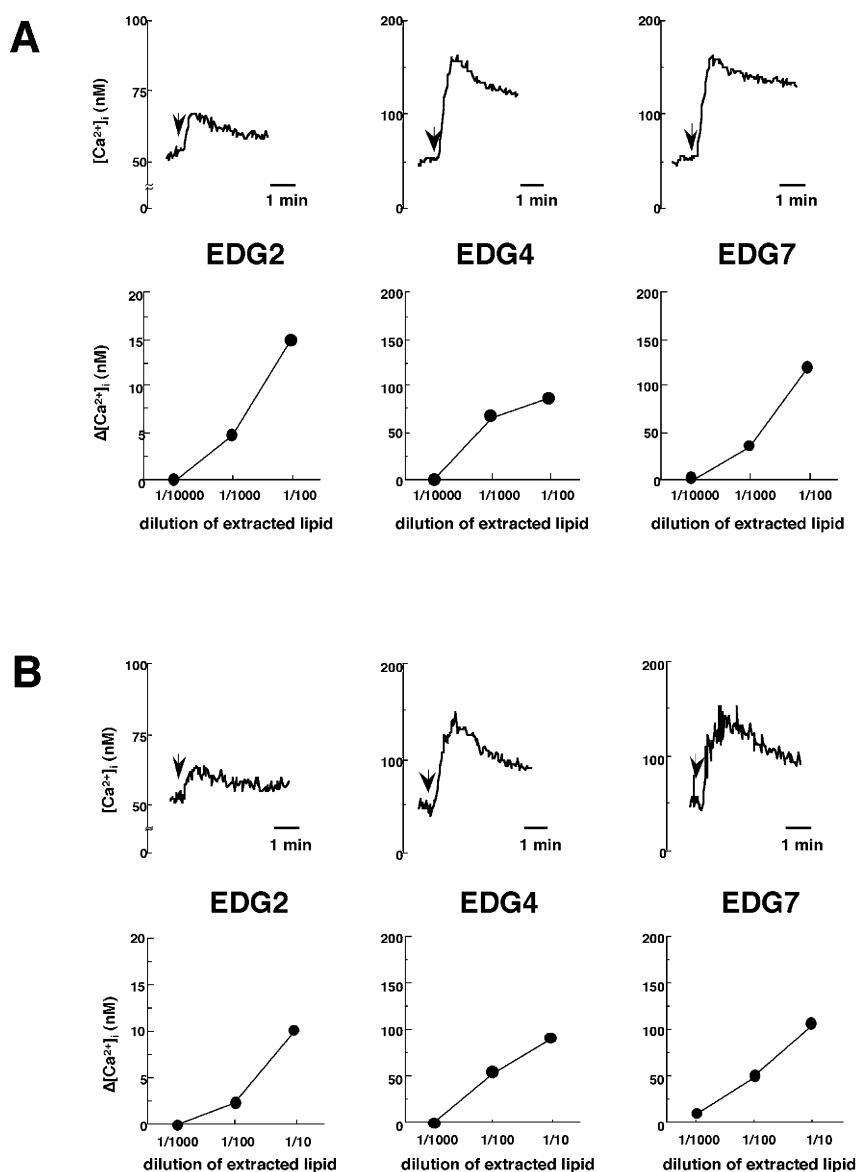
Fig. 2. LPA in biological fluids restores activities after extraction by organic solvents. Lipid extracts from incubated blood plasma of SDR (A) or seminal plasma (B) were reconstituted in buffer containing 0.1% BSA and added to Fura-2-loaded Sf9 cells expressing human EDG2, EDG4, or EDG7. LPA activities were evaluated by increases in $[Ca^{2+}]_i$ in the insect cells after samples were added to the cells. Arrows indicate the application of the diluted samples (100-fold dilution for the incubated plasma and 10-fold dilution for the seminal fluids). Dose–response curves are shown in the lower panels.

C-terminal amino acids of EDG2 (41 amino acids) were replaced with those of EDG4 (54 amino acids), and found that the chimeric receptor did transduce the LPA-induced Ca^{2+} response in the insect cells [16]. In this study, we used this chimeric receptor as EDG2.

2.4. Quantification of LPA

LPA concentration was determined by fluorometry of H_2O_2 using HPPA (7.5 mM) as a peroxidase donor [20] generated by reaction of LPA samples with 500 U/ml monoglyceride lipase (Asahi Chemical Industry, Shizuoka, Japan) and 500 U/ml glycero-3-phosphate oxidase (Asahi Chemical Industry) in a buffer containing 50 mM HEPES, 2 mM $CaCl_2$, 0.2% Triton X-100, 100 U/ml peroxidase (Toyobo, Tokyo, Japan), pH 7.5 in a total volume of 1500 μl. Fluorescence intensity at excitation at 320 nm/emission at 404 nm was measured with a fluorometer (Hitachi, Ibaraki) 5 min after mixing the samples. We detected LPA at concentrations as low as 0.1 nmol and obtained linearity up to 10 nmol of LPA in this system. To determine low concentrations of LPA, LPA in a sample was extracted by the method of Bligh and Dyer [21] under acidic conditions (pH 3.0, see below) and more samples were introduced in the assay.

2.5. Preparation of biological fluids

The biological fluids used in this study included three types of blood fluid from rats and six other types of fluid from human volunteers. Rat blood was collected by cardiac puncture. Rat serum was prepared by allowing the blood to clot for 60 min at 37°C and then centrifuging it at $2000 \times g$ for 20 min at 4°C to remove the clot. To obtain plasma, blood was drawn in the presence of one-sixth volume of acid citrate dextrose (3% citrate, 2.2% D-glucose, pH 6.0) and immediately centrifuged at $2000 \times g$ for 20 min at 4°C to remove the cells. The third blood fluid used in the assays was incubated plasma, which was obtained by incubating plasma at 37°C for 60 min. The human fluids were collected from healthy men between the ages of 22 and 26. Seminal fluids ($n = 4$) were kept at room temperature for 30 min after collection and then mixed with an equal volume of phosphate-buffered saline. Seminal plasma was prepared by centrifuging the seminal

fluid at $500 \times g$ for 10 min at room temperature to remove the sperm. LPA in the seminal plasma was extracted by the method of Bligh and Dyer [21] under acidic conditions (pH 3.0). The other human biological fluids were saliva, urine, tears, sweat, and nasal mucus ($n = 3$), and were used immediately after collection.

2.6. Lipid extraction

Phospholipids were extracted by the method of Bligh and Dyer [21] under acidic conditions by adjusting the pH to 3.0 with 1 N HCl. Lipids in the aqueous phase were re-extracted and pooled with the previous organic phase. The extracted lipids were dried, dissolved in chloroform/methanol (1:1) and used for the bioassays. The recovery of lipids was monitored by the addition of trace amounts of 1-[^{3}H]oleoyl-LPA to the samples. Under the above conditions, recovery of 1-[^{3}H]oleoyl-LPA was always $> 95\%$.

3. Results and discussion

3.1. Detection of LPA in biological fluids by evaluating the activation of LPA receptors

We searched for LPA activity in various biological fluids by measuring the LPA-induced change in intracellular calcium concentration ($[Ca^{2+}]_i$) in three types of Sf9 insect cells, each expressing one of the LPA receptors (EDG2, EDG4, and EDG7). We previously showed that each LPA receptor expressed in the Sf9 cells was strongly activated by exogenously added sub-nanomolar to nanomolar concentrations of LPA [8,16]. In addition, the $[Ca^{2+}]_i$ of intact Sf9 cells or cells infected with wild-type baculovirus was not affected by addition of various biological fluids (data not shown), which enabled us to measure LPA activity in the biological fluids with high sensitivity. To evaluate the activation of LPA receptors, we expressed each recombinant receptor in Sf9 cells using a baculovirus system and measured the increases in the $[Ca^{2+}]_i$ evoked by addition of biological fluids or LPA. Among the biological fluids tested (serum, plasma, incubated plasma, saliva, urine, tears, sweat, nasal mucus, and seminal plasma), serum and incubated plasma contain LPA abundantly [11,22]. Consistent with this, addition of incubated blood plasma (Fig. 1A, upper panel) induced a rapid increase in the $[Ca^{2+}]_i$ in Sf9 cells expressing EDG2 and EDG4. However, the increase in the $[Ca^{2+}]_i$ was not observed at all in Sf9 cells expressing EDG7. Similar results were obtained when serum was added to the cells (data not shown). In contrast, seminal plasma induced an EDG7-specific response (Fig. 1B, upper panel). The dose dependences of both blood and seminal plasma (Fig. 1A,B, lower panels) confirmed that the two biological fluids differentially activate LPA receptors.

3.2. Extracted LPA activates all LPA receptors

The preceding results show that different biological fluids activate different receptors. One explanation for this is that different biological fluids have different types of LPAs, because LPAs with different acyl chains and acyl positions have been shown to differentially activate the LPA receptors [8,16]. Another possibility is that the biological fluids have one or more water-soluble components that associate with the LPA and influence its activity. To test these possibilities we extracted LPA with organic solvents and dissolved it in buffer containing 0.1% BSA, and used it in the Ca^{2+} assay. As shown in Fig. 2, LPA from both blood (Fig. 2A) and seminal plasma (Fig. 2B) activated all LPA receptors. These results support the latter possibility, i.e. that the differential activa-
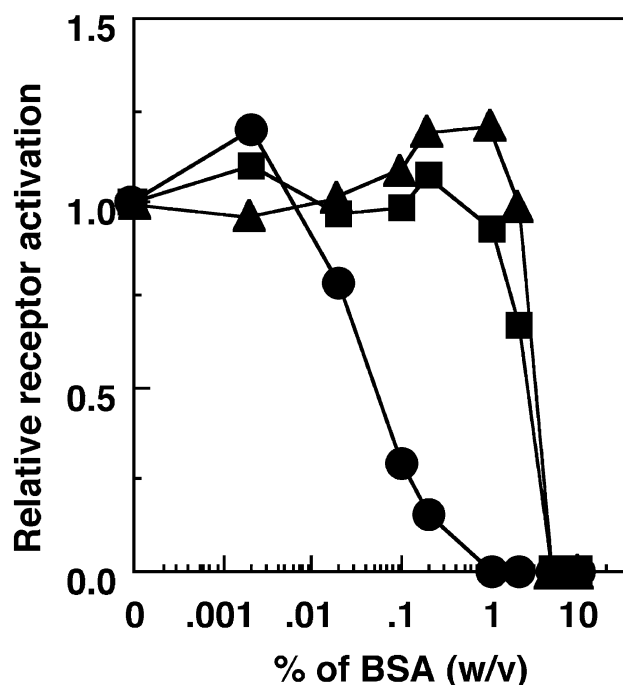

Fig. 3. Serum albumin differentially modifies the activity of LPA toward different LPA receptors. The effect of albumin on the activation of LPA receptors induced by 1-oleoyl-LPA (100 nM) was examined by measuring the transient increase in $[Ca^{2+}]_i$ in Fura-2-loaded Sf9 cells expressing human EDG2, EDG4, or EDG7. Closed triangles, squares and circles represent activation of EDG2, EDG4 and EDG7, respectively. Activations are expressed relative to the activation of each receptor when the albumin concentration is 0%.

tion of the receptors is due to one or more water-soluble components in the biological fluids.

3.3. Effect of serum albumin on the activation of LPA receptors

LPA in serum is almost exclusively associated with serum albumin [11]. In the course of this study we found that the albumin concentration in the buffer used in the Ca^{2+} assay is critical for the activation of LPA receptors. As shown in Fig. 3, high concentrations of BSA ($> 5\%$, w/w) completely inhibited activation of the three LPA receptors induced by 100 nM LPA. Among the three receptors, EDG7 was most sensitive to the albumin concentration. 0.01% BSA inhibited EDG7 activation and 1% BSA completely blocked it. However, BSA concentrations of 0.01% and 1% had no effect on EDG2 or EDG4 (Fig. 3).

To further evaluate the role of albumin in LPA receptor activation, we utilized the NAR, a mutant strain in which normal splicing of albumin mRNA does not occur. While the plasma albumin concentration is 19.4 mg/ml in normal SDR, it is only 0.4 mg/ml in NAR. The LPA levels in incubated plasma from these two rat strains were comparable, being 10.3 ± 2.1 μM ($n = 5$) in NAR and 8.9 ± 2.3 μM ($n = 5$) in SDR. Consistent with this, we did not observe any difference in activation of EDG2 and EDG4 in the two rat strains (Figs. 1A and 4A). In contrast, in the activation of EDG7, we found that plasma from NAR induced a significant Ca^{2+} response (Fig. 4A), whereas plasma from control animals (SDR) induced a poor response (Fig. 1A).

The weak effect of SDR plasma on EDG7 activation may be due to the composition of the LPA species, as discussed
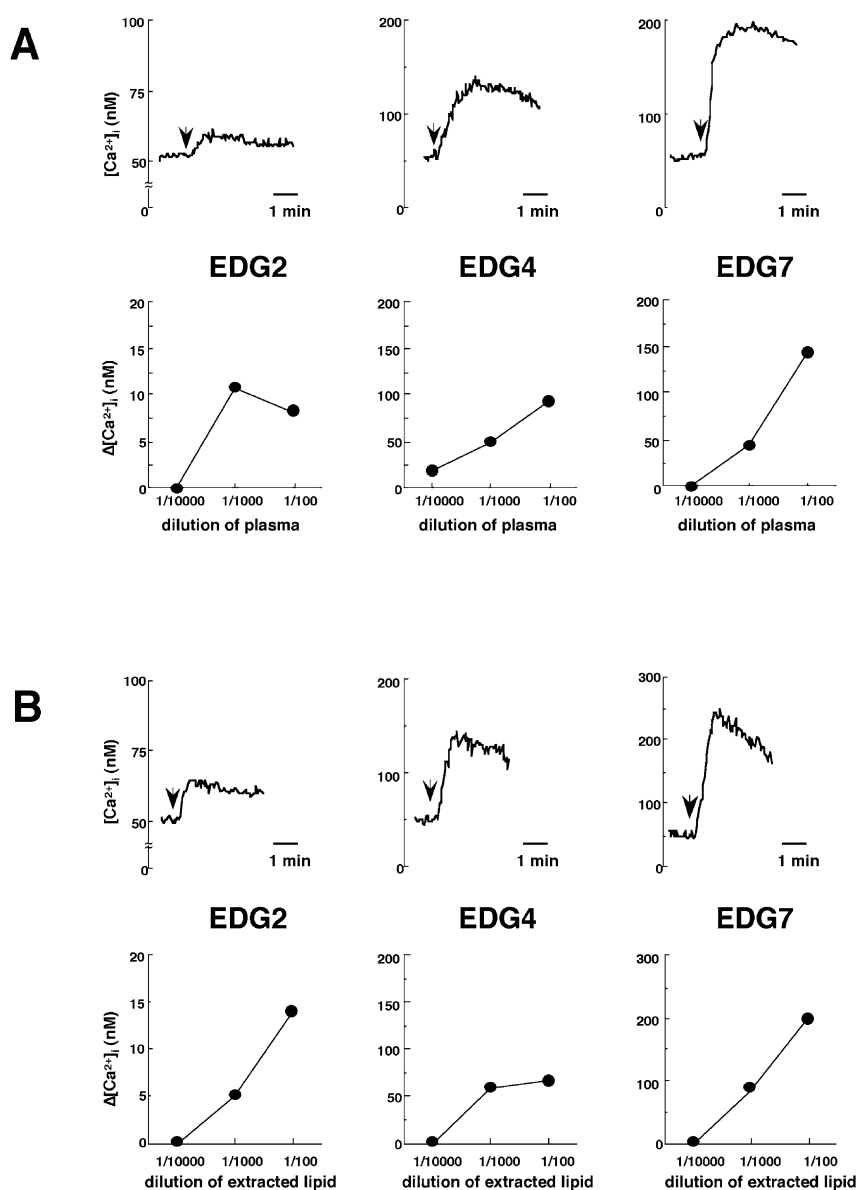
Fig. 4. Enhanced activation of EDG7 by blood plasma from NAR. Incubated blood plasma prepared from NAR (A) or lipids extracted from the plasma (B) were added to Fura-2-loaded Sf9 cells expressing human EDG2, EDG4, or EDG7. LPA activities were evaluated by increases in $[Ca^{2+}]_i$ in insect cells after samples were added to the cells. Arrows indicate the application of 100-fold diluted samples. Dose–response curves are shown in the lower panels.

above. To test this possibility we examined the activity of the extracted LPA. After extraction, the lipid fractions from NAR (Fig. 4B) and SDR (Fig. 2B) showed almost equal abilities to activate EDG7, EDG2, and EDG4. Furthermore, addition of BSA to NAR plasma weakened the activation of EDG7 (data not shown). Thus, it can be concluded that the disappearance of the major serum protein (albumin) but not the composition of the acyl chain of LPA is responsible for the enhanced reactivity of EDG7 to NAR plasma.

It is generally believed that serum albumin has some physiological role in regulating the activities of lysophospholipid mediators including platelet-activating factor, sphingosine 1-phosphate, lysophosphatidylcholine, and LPA, because the biological activities of these compounds are dependent on albumin concentration. Actually, LPA, which is present at micromolar concentrations in serum, forms a complex with albumin [11,23]. In this study we clearly demonstrated that

the albumin concentration is critical for the activation of LPA receptors in a receptor type-specific manner. We observed that (1) higher concentrations of albumin (1–5%) suppressed the activation of EDG7, but not the activation of EDG2 or EDG4 (Fig. 3) and (2) plasma from NAR but not from control SDR activated EDG7 (Fig. 4). Together, these results clearly show that activation of EDG7 does not occur in the presence of physiological concentrations of albumin, since the albumin concentration in blood plasma is normally about 4% (w/v). Furthermore, we detected LPA activity in seminal plasma and found that EDG7 was selectively activated by the LPA (Fig. 1). The seminal plasma could not activate either EDG2 or EDG4. However, when the LPA was extracted from the seminal plasma with an organic solvent and reconstituted in buffer containing albumin, its activity toward EDG2 and EDG4 was recovered. This strongly indicates that seminal plasma has one or more carrier proteins

that efficiently deliver LPA to EDG7 but not to the other two receptors. The carrier proteins are different from albumin, because the effect of LPA bound to albumin on activating LPA receptors was opposite to the effect to the LPA detected in seminal plasma. The albumin concentration in seminal plasma is very low (0.063%, w/v) [24]. This concentration does not inhibit the activation of EDG7 (Fig. 3), which is consistent with the selective activation of EDG7 by seminal fluid. In addition to serum albumin, several cellular proteins, such as fatty acid-binding protein [25] and an actin-severing protein, gelsolin, have been characterized as LPA-binding proteins. Like albumin, gelsolin is also present in plasma ($\sim$100 µg/ml). Recently it was demonstrated that gelsolin enhances the effects of LPA on cellular functions in rat cardiac myocytes by binding to LPA [18]. If seminal plasma does have a protein factor, it may differ from gelsolin, because cardiac myocytes express both EDG2 and EDG4 [18], which are not activated by LPA in seminal plasma (Fig. 1). EDG2 and EDG4 are widely distributed in both human and mouse tissues, whereas EDG7 is distributed predominantly in reproductive tissues such as testis, prostate, and ovary [8,9]. In addition, EDG7 appears to be expressed by epithelial cells in human prostate (our unpublished result). These cells are exposed to seminal plasma but not to blood plasma. The expression pattern of EDG7 also supports the hypothesis that EDG7 has a role in reproductive tissues where the albumin concentration is low.

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