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Disintegration of Lysosomes Mediated by GTP γ S-Treated Cytosol: Possible Involvement of Phospholipases¹

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We showed previously that cytosol treated with guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) disintegrated lysosomes *in vitro* [Sai, Y. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 198, 869-877] in time-, temperature-, and dose-dependent manners. This also requires ATP, however, the latter can be substituted with deoxy-ATP, ADP, or ATP γ S, suggesting no requirement of ATP hydrolysis. The lysis was inhibited by several chemical modifiers, including *N*-ethylmaleimide, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, and by various phospholipase inhibitors (trifluoperazine, *p*-bromophenacyl bromide, nordihydroguaiaretic acid, W-7, primaquine, compound 48/80, neomycin, and gentamicin), but not by ONO-RS-082, an inhibitor of phospholipase A₂. The reaction was also inhibited by phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidic acid, and phosphatidylcholine) and diacylglycerol. Among the phospholipase A₂ hydrolysis products of phospholipids, unsaturated fatty acids (oleate, linoleate, and arachidonate) and lysophospholipid (lysophosphatidylcholine) by themselves broke lysosomes down directly, whereas saturated fatty acids (palmitate and stearate) had little effect. We found that GTP γ S-stimulated cytosolic phospholipase A₂ activity was highly sensitive to ONO-RS-082. These results suggest the participation of phospholipase(s), though not cytosolic phospholipase A₂, in the GTP γ S-dependent lysis of lysosomes.

Key words: G-protein, GTP γ S, lysis, lysosome, phospholipase A₂.

We previously identified distinct subsets of small GTP-binding proteins on rat liver lysosomal membranes (1), and recently established a novel *in vitro* assay system to assess the possible role of GTP-binding proteins in the lysis of lysosomes, *i.e.*, disintegration of isolated lysosomes by GTP γ S-treated cytosol (2). The effect of GTP γ S was

reversed by GTP and GDP. This suggests the participation of G-protein(s) in the lysis. GTP γ S-dependent lysosomal lysis involves two steps, (i) activation of the G-protein by GTP γ S and (ii) ATP-dependent disintegration of lysosomes by the activated G-protein.

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A number of substances are known to act as lysosome stabilizers, including lipoprotein lipase, lysozyme, phospholipase A₂ (PLA₂) (3), and free radicals (4, 5). The some of activity PLA₂ is stimulated by GTP γ S (6, 7), and the superoxide-generating NADPH oxidase system in phagocytes is stimulated by a small G-protein, rac p21 (8).

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Abbreviations: AMP-PPP, β , γ -methyleneadenosine 5'-triphosphate; AMP-PCP, α , β -methyleneadenosine 5'-triphosphate; AMP-PNP, β , γ -imidoadenosine 5'-triphosphate; ATP γ S, adenosine 5'-O-(3-thiotriphosphate); CAD, cationic amphiphilic class drug; DAG, diacylglycerol; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; ER, endoplasmic reticulum; FD, fluorescein isothiocyanate-dextran; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); LysoPC, lysophosphatidylcholine; MAG, monoacylglycerol; MOPS, 3-[*N*-morpholino]-propane sulfonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NDGA, nordihydroguaiaretic acid; NEM, *N*-ethylmaleimide; ONO-RS-082, 2-(*p*-amylcinnamonyl)amino-4-chlorobenzoic acid; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PI, phosphatidylinositol; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; SVE, 0.25 M sucrose, 1 mM EDTA (pH 7.0), and 0.1 (v/v)% EtOH; TMAH, tetramethylammonium hydroxide.

Recently, it was reported that small G-proteins participate in vesicular transport. This process involves a series of highly coordinated and specific membrane fusion events. However, the role of G-proteins in lysosomal function remains to be clarified. Our novel *in vitro* assay system might reflect some essential unit reaction related to lysosomal dynamics involving GTP γ S and may constitute a good *in vitro* model system for exploring the role of G-proteins in lysosomal function.

The aims of the present study are to characterize the lysis reaction in detail, to examine the possible participation of phospholipases, and to determine the relevance of the lysis to membrane fusion.

MATERIALS AND METHODS

Materials—Fluorescein isothiocyanate-dextran (FD; Av. Mol. Wt.: 70,000) and Percoll were purchased from Sigma

(St. Louis) and Pharmacia (Uppsala), respectively. Protease inhibitors of microbial origin (pepstatin A, chymostatin, antipain, and leupeptin) were obtained from the Peptide Research Institute (Osaka). Most other chemicals were obtained from Sigma (St. Louis).

Preparation of Lysosomes—Dextran-filled lysosomes (dextranosomes) were prepared essentially as described (9) with the exception that the Percoll-washout procedure was omitted and the lysosomal layer in the Percoll gradient was used for the experiments.

Preparation of Cytosols—The livers from overnight fasted rats were homogenized at 0°C in 3 volumes of SVE [0.25 M sucrose, 1 mM EDTA, pH 7.0, 0.1% (v/v) EtOH] containing 5 $\mu\text{g}/\text{ml}$ of each protease inhibitor (pepstatin A, chymostatin, antipain, and leupeptin). The homogenate was centrifuged at $1,250\times g$ for 8 min and the resulting supernatant was pooled. The precipitate was homogenized again in the same buffer and then centrifuged at $550\times g$ for 11 min, and the resulting supernatant was combined with the first supernatant. This procedure was repeated twice. The combined supernatant was centrifuged at $100,000\times g$ for 1 h, and the resulting supernatant (5–10 mg protein/ml) was frozen in liquid N_2 and stored at -80°C until use.

Lysis Assay—Unless otherwise indicated, the assay mixture (1 ml) comprised dextranosomes (20–50 μg protein), cytosol [either non-treated (350 μg protein) or pretreated at 37°C for 10 min with $GTP\gamma S$ (100 μM), 350 μg protein], 1 mM ATP and 100 nM baflomycin A_1 , in 0.1 M KCl, 0.2 M sucrose, 10 mM $MgCl_2$, and 20 mM 3-[*N*-morpholino]propane sulfonic acid (MOPS)-tetramethylammonium hydroxide (TMAH) (pH 7.0) (5 μM $GTP\gamma S$ carried into the assay medium). After 20 min at 37°C , intact lysosomes were sedimented by centrifugation at $8,600\times g$ for 5 min through 50 μl of silicon oil (Shin-Etsu Chemical, KF-54), which had been layered at the bottom of the tube. Lysosomal lysis was estimated fluorometrically by measuring the release of FD from lysosomes and expressed as a percentage of the total FD obtained on the

treatment of lysosomes with 0.1% Triton X-100. A correlation was observed between the release of FD from lysosomes and that of β -*N*-acetylglucosaminidase (lysosomal enzyme). All data shown are representative of repeated experiments performed with separate preparations of lysosomes.

Assaying of Phospholipase A_2 —Enzyme activity was assayed in 250 μl of buffer comprising 20 mM MOPS/TMAH (pH 7.0), 0.1 M KCl, 0.2 M sucrose, 2 mg/ml BSA, and 10 mM $MgCl_2$. Radiolabeled *L*- α -1-palmitoyl, 2-arachidonoyl, [arachidonoyl-1- ^{14}C]phosphatidylethanolamine (0.5 nmol, 57 mCi/mmol) was included as a substrate in the reaction mixture, a liposomal suspension obtained by sonication (5 min in the buffer). The reaction was started by adding the enzyme, carried out at 37°C for 30 min and stopped by adding 1.25 ml of Dole reagent [isopropanol/*n*-hexane/1 N sulfuric acid = 70 : 20 : 2% (v/v)]. The released fatty acids were extracted with 0.75 ml *n*-heptane containing 100 mg silica gel added for the absorption of phospholipids. The supernatant containing the released arachidonic acid was obtained by centrifugation at 2,500 rpm for 5 min and its radioactivity was measured with a liquid scintillation counter.

RESULTS

Disintegration of Lysosomes Induced by $GTP\gamma S$ -Activated Cytosol—The integrity of lysosomes isolated from rat liver was estimated by measuring the release of the lysosomal contents (fluorescein-dextran, FD) in the presence of $GTP\gamma S$ -treated or non-treated cytosol over broad time and temperature ranges. $GTP\gamma S$ -treated cytosol broke lysosomes down in a dose-dependent manner in the range of 10–500 μg protein/ml, the optimum concentration being 500 μg protein/ml (Fig. 1). Non-treated cytosol did not show any effect regardless of the concentration. Figures 2 and 3 show the time-course and temperature-dependency of this reaction. Disintegration of lysosomes took place time-dependently with a lag of about 10 min and reached 60% in 60 min. Very little lysis was observed below 25°C , and a steep increase occurred between 25 and 37°C . $GTP\gamma S$ -dependent disintegration of lysosomes required

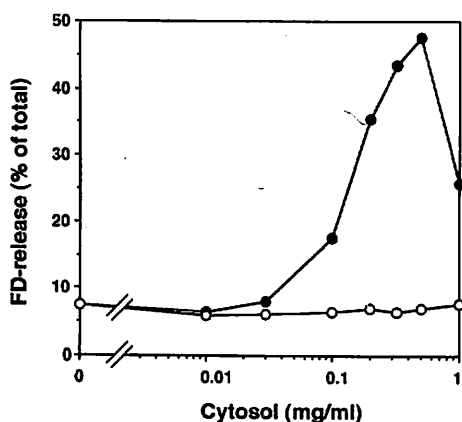


Fig. 1. Dose-dependent effect of cytosol on lysosomal lysis. Lysosomes isolated from rats given FD were incubated with increasing amounts of either untreated (○) or $GTP\gamma S$ -treated (●) cytosol for 20 min at 37°C in the presence of 1 mM ATP and 100 nM baflomycin A_1 . Intact lysosomes were sedimented and the FD released from the lysosomes was estimated fluorometrically as described under "MATERIALS AND METHODS." Values shown are representative of three experiments performed with three separate preparations of lysosomes and cytosol.

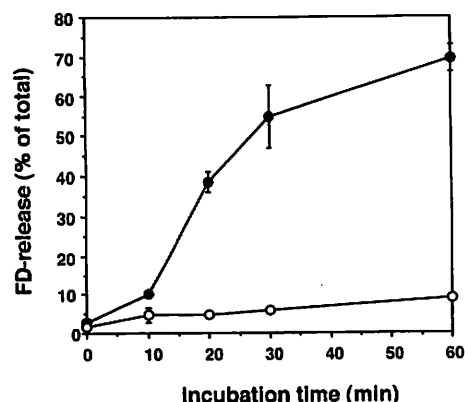


Fig. 2. Time-course of lysosomal lysis. Lysosomal lysis was measured over a long period of time with either untreated (○) or $GTP\gamma S$ -treated (●) cytosol at 37°C as described under "MATERIALS AND METHODS." Values shown are the averages and ranges of duplicates, and are representative of two experiments.

very high activation energy (42.7 kcal/mol), as estimated from Arrhenius plots (Fig. 3, insert). This probably suggests a mechanism involving reorganization of membrane phospholipids that is usually associated with highly temperature-dependent reactions of membrane fusion and/or fission (10). This is also in agreement with its strong inhibition by polyene antibiotics, nystatin and amphotericin B, both of which bind to cholesterol in membranes and

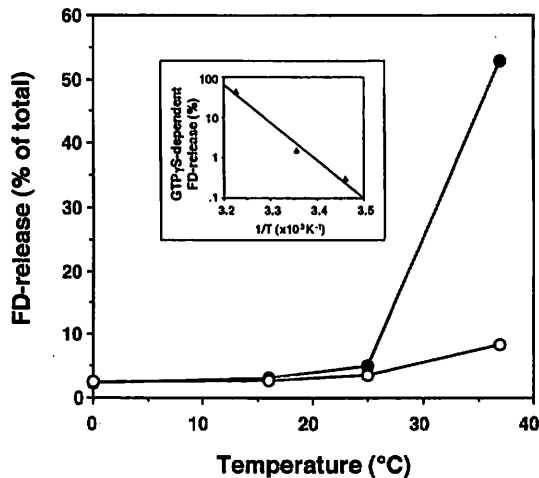


Fig. 3. Temperature-dependence and Arrhenius plots (insert) of lysosomal lysis. Lysosomal lysis was measured after 20 min incubation at 0, 16, 25, or 37°C with either untreated (○) or GTP γ S-treated (●) cytosol as described under "MATERIALS AND METHODS." Arrhenius plots were obtained for GTP γ S-dependent FD-release (FD-release by GTP γ S-treated cytosol minus that by control cytosol) vs. $1/T$. Values shown are representative of two experiments performed with two separate lysosome preparations.

TABLE I. Nucleotide specificity of GTP γ S-dependent disintegration of lysosomes. GTP γ S-dependent disintegration of lysosomes (GTP γ S-dependent lysosomal lysis) was measured in the absence or presence of the indicated nucleotides at a concentration of 1 mM as described under "MATERIALS AND METHODS," and expressed as % FD-release from lysosomes by GTP γ S-treated cytosol minus that by control cytosol.

| Nucleotides | GTP γ S-dependent FD-release (% of control) |
|--------------------|----------------------------------------------------|
| ATP [control] | 100 |
| dATP | 109 |
| ADP | 106 |
| ATP γ S | 69.4 |
| AMP-CPP | 31.0 |
| AMP-PNP | 20.4 |
| AMP-PCP | 4.6 |
| AMP | 6.5 |
| cAMP | 6.1 |
| GTP | 51.4 |
| ITP | 16.5 |
| UTP | 13.4 |
| CTP | 15.9 |
| GDP | 1.4 |
| PP _i | 2.8 |
| None | 1.2 |
| ATP+AMP | 105 |
| ATP+AMP-PNP | 83.9 |
| -MgCl ₂ | 0 |
| +1 mM EDTA | -1.0 |

restrict membrane fluidity.

ATP Requirement of GTP γ S-Dependent Lysis—Table I shows the characterization of the reaction in terms of ATP requirement, nucleotide specificity and the effects of certain ATPase inhibitors (Table II). Among the nucleotides, not only ATP but also deoxy-ATP and ADP were able to induce significant lysis. ATP γ S and GTP, although much less effective, also supported the lysis. AMP-CPP, AMP-PNP, ITP, UTP, and CTP partially supported the lysis, but AMP, cAMP, AMP-PCP, GDP, and pyrophosphate (PP_i) did not. Neither AMP nor AMP-PNP could compete with the effect of ATP at 1 mM. The activity was dependent on Mg²⁺. These findings suggest that binding but not hydrolysis of ATP is required for this reaction.

Among the ATPase inhibitors, neither vanadate (for P-type ATPases), azide nor oligomycin (both for F-type ATPases) was inhibitory, although quercetin (low specificity inhibitor) was partially inhibitory (Table II). It should be noted that baflomycin A₁, a potent selective inhibitor of V-type ATPases, was included in all assay media in this study. These results suggest that none of the H⁺-ATPases participate in this reaction.

Effects of Various Chemical Modifiers and Inhibitors—To characterize the nature of the GTP γ S-dependent lysosomal lysis, a series of inhibitors and known chemical

TABLE II. Effects of ATPase inhibitors on GTP γ S-dependent lysosomal lysis. GTP γ S-dependent lysis was measured in the absence or presence of the indicated inhibitors as described under "MATERIALS AND METHODS." Values shown are representative of two experiments performed with two separate preparations of lysosomes.

| Inhibitors | GTP γ S-dependent FD-release (% of control) |
|-----------------------------|----------------------------------------------------|
| None [control] | 100 |
| Sodium vanadate (1 mM) | 95.6 |
| Sodium azide (1 mM) | 89.4 |
| Oligomycin (2.5 μ g/ml) | 82.6 |
| Quercetin (20 μ M) | 49.7 |

TABLE III. Effects of various chemicals on GTP γ S-dependent lysosomal lysis. GTP γ S-dependent lysis was measured in the absence or presence of the indicated chemicals as described under "MATERIALS AND METHODS." Values shown are representative of two experiments performed with two separate preparations of lysosomes.

| Chemical modifiers | GTP γ S-dependent FD-release (% of control) |
|-------------------------------------|----------------------------------------------------|
| None [control] | 100 |
| N-Ethylmaleimide (1 mM) | -5.6 |
| NBD-Cl (100 μ M) | 2.8 |
| DIDS (100 μ M) | 2.5 |
| Diethylpyrocarbonate (1 mM) | 58.5 |
| 2,3-Butanedione (10 mM) | 86.8 |
| N-Bromosuccinimide (10 μ M) | 106 |
| Dithiothreitol (0.1 mM) | 99.7 |
| Superoxide dismutase (300 units/ml) | 76.6 |
| Catalase (1,500 units/ml) | 83.8 |
| Mannitol (20 mM) | 93.2 |
| Histidine (20 mM) | 111 |
| Colchicine (50 μ M) | 94.8 |
| Cytochalasin B (1 μ M) | 101 |
| Amphotericin B (250 μ g/ml) | 27.8 |
| Nystatin (200 μ g/ml) | 7.8 |

modifiers of proteins were tested. Among them, diethylpyrocarbonate partially (41% inhibition at 1 mM), *N*-ethylmaleimide (NEM), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) effectively inhibited the lysis (Table III).

Neither scavengers of active oxygen species, including superoxide dismutase (for O₂⁻), catalase (for H₂O₂), mannitol (for \cdot OH), and histidine (for ¹O₂), nor microtubule disrupting agents, including colchicine and cytochalasin B, affected the lysosomal lysis, suggesting that the reaction was not caused by active oxygen species or mediated by cytoskeletal activity.

The effects of typical phospholipase inhibitors are summarized in Table IV. Cationic amphiphilic class drugs (CADs), such as *p*-bromophenacyl bromide, propranolol, chloroquine, nordihydroguaiaretic acid (NDGA), trifluo-

TABLE IV. Effects of phospholipase inhibitors on GTP γ S-dependent lysosomal lysis. GTP γ S-dependent lysis was measured in the absence or presence of the indicated inhibitors as described under "MATERIALS AND METHODS." Values shown are representative of two to three experiments performed with two to three separate preparations of lysosomes. N.I., no inhibition up to 300 μ M.

| Inhibitors | IC ₅₀ |
|---------------------------------|----------------------------|
| <i>p</i> -Bromophenacyl bromide | 19 μ M |
| Propranolol | 200 μ M |
| NDGA | 34 μ M |
| Chloroquine | 1 mM |
| Trifluoperazine | 8.3 μ M |
| W-7 | 48 μ M |
| Primaquine | 200 μ M |
| Compound 48/80 | 190 ng/ml |
| Gentamicin | 2 mM |
| Amikacin | 80 mM |
| Neomycin | 60 μ M |
| Spermine | 400 μ M |
| ONO-RS-082 | N.I. |
| EtOH | 35% inhibition at 1% (v/v) |

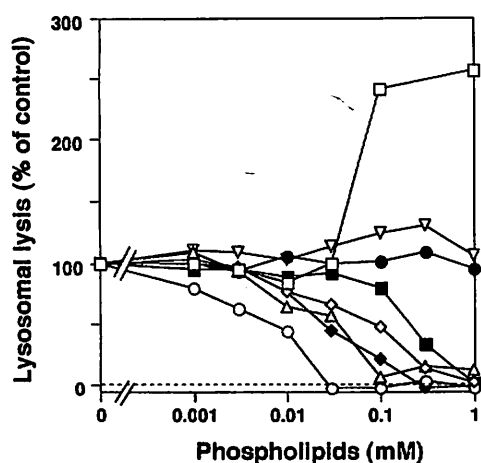


Fig. 4. Effect of phospholipids on lysosomal integrity. Lysosomal lysis was measured in the presence of PC (\diamond), PI (\circ), PS (\blacklozenge), PA (\triangle), lysoPC (\square), DAG (\blacksquare), ceramide (\bullet), or sphingomyelin (∇), as described under "MATERIALS AND METHODS." Values shown are representative of two experiments performed with two separate lysosome preparations. The lysosomal lysis is shown as a percentage of a control that was incubated with GTP γ S-treated cytosol.

perazine, W-7, primaquine, and compound 48/80, have inhibitory effects on various phospholipases (11). All these molecules have both distinct polar and nonpolar moieties, and tend to interact with membrane surfaces. All showed inhibitory activity toward GTP γ S-dependent lysosomal lysis at comparable doses. At sufficiently high concentrations, some of these amphiphilic molecules exhibited a detergent-like action and thus disrupted the lysosomes. Optimal inhibition was only observed in a limited range of concentrations for trifluoperazine, W-7 and compound 48/80; at high concentrations, they caused non-specific (GTP γ S-independent) lysis (data not shown). Among the amphiphilic molecules, polyene antibiotics, nystatin, and amphotericin B, showed strong inhibition of the GTP γ S-dependent lysis. All the molecules combine with cholesterol

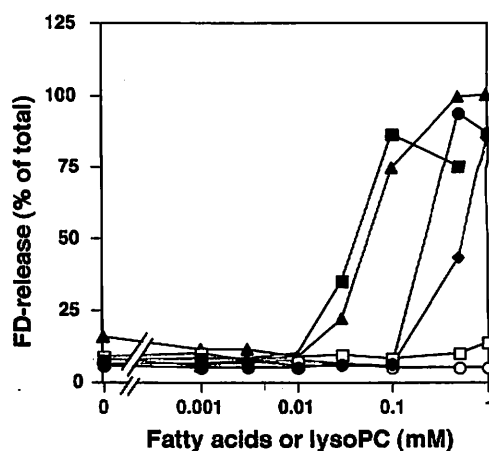


Fig. 5. Effects of fatty acids and a lysophospholipid on lysosomal integrity. Lysosomes were incubated with increasing amounts of either fatty acids [arachidonate (\blacksquare), oleate (\blacklozenge), linoleate (\bullet), palmitate (\circ), and stearate (\triangle)] or a lysophospholipid [lysophosphatidylcholine (\blacktriangle)] in the presence of 350 μ g/ml of non-treated cytosol, 1 mM ATP, and 100 nM bafilomycin A₁. FD released from lysosomes was estimated as described in the legend to Fig. 1. Values shown are representative of two experiments performed with two separate lysosome preparations.

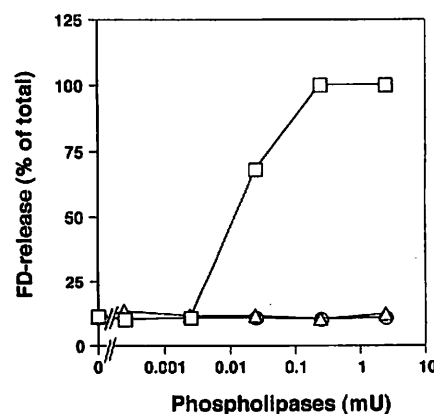


Fig. 6. Effects of phospholipase A₂, C, and D on lysosomal integrity. Lysosomes were examined with various amounts of phospholipases [phospholipase A₂ (\square), C (\triangle), and D (\circ)] for 30 min at 37°C. FD released from lysosomes was estimated as described in the legend to Fig. 1.

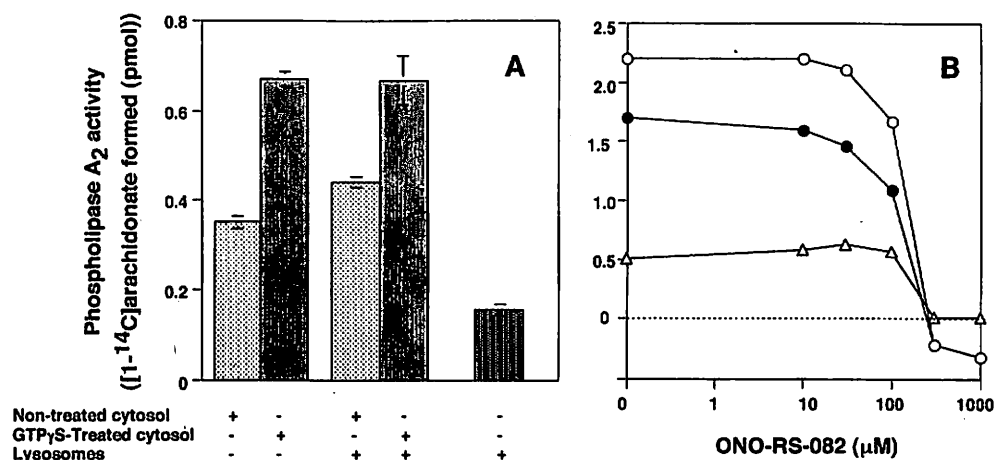


Fig. 7. Phospholipase A₂ activities of GTP γ S-treated and non-treated cytosol and lysosomes (A), and their sensitivity to ONO-RS-082 (B). The phospholipase A₂ activities of GTP γ S (300 μ M)-treated and non-treated cytosol (200 μ g) and lysosomes (32 μ g) were assayed as described under "MATERIALS AND METHODS." In B, the phospholipase A₂ activities of GTP γ S-treated cytosol (○) and non-treated cytosol (Δ) were both measured in the presence of lysosomes, and the differences (●) were plotted.

in biological membranes and form pores. But the concentrations required for pore formation are much higher than those for the inhibition of lysosomal lysis. Aminoglycoside antibiotics, gentamicin and amikacin, inhibit lysosomal phospholipases A₂ and C (12), and both neomycin and spermine inhibit phospholipase C (PLC). All significantly inhibited the GTP γ S-dependent lysis. But ONO-RS-082, an inhibitor of PLA₂, was ineffective. In the presence of a primary alcohol, PLD produces phosphatidylalcohol instead of PA (13). Therefore, if the production of PA by PLD is critical for subsequent events in GTP γ S-dependent lysis, the presence of an alcohol during PLD activation should inhibit the lysis. As shown in Table IV, 1% (v/v) EtOH partially blocked the lysis (35% inhibition).

Effects of Phospholipids and Fatty Acids—Cationic amphiphilic drugs exhibit particularly high binding activity toward anionic lipids. It is hypothesized that the resulting drug-phospholipid complexes resist hydrolysis (14). To further confirm the participation of phospholipases in the GTP γ S-dependent lysis of lysosomes, the effects of various lipids and fatty acids were examined. Among the phospholipids, phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylcholine (PC) strongly, and diacylglycerol (DAG) moderately inhibited the lysis, whereas sphingomyelin and ceramide showed no significant effect up to 1 mM (Fig. 4). Contrary to these results, lysoPC alone disintegrated lysosomes. The effects of fatty acids on lysosomal integrity are shown in Fig. 5. Unsaturated fatty acids [oleate (18 : 1), linoleate (18 : 2), and arachidonate (20 : 4)] broke lysosomes down, whereas none of the saturated fatty acids [palmitate (16 : 0) and stearate (18 : 0)] had an effect up to 1 mM.

Cis-unsaturated fatty acids induce membrane fusion *in vivo*, including in secretory granules (15, 16). LysoPC and free fatty acids generated by PLA₂ are both membranolytic agents (17, 18). Fatty acid metabolites generated by PLA₂ play a critical role in the regulation of lysosomal enzyme release from human polymorphonuclear leukocytes (19), and lysoPC is generally considered to be a putative biological fusogen (20).

Effects of PLA₂, PLC, and PLD—To determine whether or not the hydrolysis of endogenous phospholipids is important for lysosomal lysis, we added phospholipases to the assay mixture. The addition of PLA₂ (1 mU) (from *Naja naja*), but not PLC (from *Clostridium welchii*) or PLD

(from *Streptomyces chromofuscus*) (up to 2.5 mU), induced lysis (Fig. 6).

Activation of Cytosolic PLA₂ by GTP γ S—The observations described above suggest that, among various phospholipases, PLA₂ plays an important role in the GTP γ S-dependent lysis of lysosomes. The GTP analogs, β , γ -imidoguanosine 5'-triphosphate and GTP γ S, are potent activators of PLA₂ in permeabilized neutrophils and HL60 cells, and it was reported that pretreatment of homogenates of HL-60 granulocytes with specific antibodies against cytosolic PLA₂ attenuated the basal as well as stimulated arachidonate release (21). The presence of PLA₂ activity in lysosomes has also been reported (22). Therefore, cytosolic or lysosomal PLA₂ might well be regulated by G proteins. In fact, in our experiments, GTP γ S significantly stimulated the activity of cytosolic PLA₂, but not that of lysosomal PLA₂ (Fig. 7A). However, the GTP γ S-stimulated PLA₂ was totally inhibited by ONO-RS-082, a PLA₂ inhibitor which does not inhibit GTP γ S-stimulated lysis (Fig. 7B). These results suggest that cytosolic PLA₂ does not participate in the lysis.

DISCUSSION

In this study, we showed that disintegration of rat liver lysosomes induced by GTP γ S-activated cytosol proceeds in time-, temperature-, and dose-dependent manners. This reaction seems to be specific for lysosomes, because lysosomes isolated from guinea pig liver also showed significant lysis, whereas no other organelle tested, including mitochondria, peroxisomes, and Golgi from rat liver, granules from guinea pig neutrophils, and red blood cells from rat, showed any lysis (data not shown).

The results of the inhibitor study indicate the possible participation of phospholipases in the labilization of lysosomes. The agents that inhibited the GTP γ S-dependent lysis have widely varying pharmacological properties, but share cationic amphiphilic features and inhibit phospholipases (A₂, C, and D). These cationic amphiphilic agents generally bind strongly to acidic phospholipids such as PS and PI. Neomycin and amikacin also bind to PI and PS, respectively, thereby inhibiting phospholipases indirectly, not through interaction with the enzymes. These results suggest that phospholipid catabolism in lysosomal membrane is important for GTP γ S-dependent lysis.

It is unlikely that the energy for the GTP γ S-dependent lysis of lysosomes is provided by ATP, because ADP and a nonhydrolyzable analog of ATP, ATP γ S, could substitute for ATP. Their nucleotide requirement shows some resemblance to that for the activation of phospholipases.

Evidence suggests that certain types of cytosolic phospholipases (PLA₂, PLC, and PLD) are activated by GTP γ S in cell-free systems as well as in semi-intact cells including human neutrophils, platelets, and mast cells (23). Several transport steps, along with the endocytic and exocytotic pathways, have been reported to be regulated by phospholipases. For example, PLA₂ stimulates the secretion of amylase from pancreatic acini (24), degranulation of rat mast cells (25), and intra-Golgi vesicular transport (26) and endosome-endosome fusion (27) *in vitro*. And PLD mediates the Golgi-coated vesicle formation dependent on ADP-ribosylation factor (small G-protein) and GTP γ S (28). Although the precise role played by lipids in the regulation of vesicular transport remains to be clarified, their importance is clear. The membrane fusion process of vesicular transport is suggested to involve several discrete steps: (i) attachment of donor membranes to the target membranes, (ii) perturbation of the membrane conformation followed by fusion pore formation, (iii) pore dilatation, and finally (iv) significant membrane mixing (29). However, little is known about the nature and origin of the force that perturbs biological membranes and finally promotes their fusion, although influenza virus fusion mediated by hemagglutinin has been relatively well characterized (30), where the generation of amphiphilic molecules (*e.g.*, lyso-phospholipids and fatty acids) has been implicated in membrane perturbation. The fusion process requires a transient conformational change of the planar membrane structure. Hence, phospholipase(s) may well regulate the fusion process.

Among the phospholipases, exogenous PLA₂, but not PLC or PLD, disintegrated lysosomes. And unsaturated fatty acids and lysoPC, products of the PLA₂ action, also induced the lysis. Moreover, we found that PLA₂ activity in rat liver cytosol was activated significantly on treatment with GTP γ S. However, both the basal and GTP γ S-stimulated cytosolic PLA₂ activities were inhibited by ONO-RS-082, which had no detectable effect on the GTP γ S-dependent lysis. This suggests that cytosolic PLA₂ does not participate in the reaction. Although exogenous PLD added to our assay system had little detectable effect on lysosomal integrity, it still remains a candidate phospholipase for lysosomal break down, because the GTP γ S-dependent reaction was inhibited by EtOH, and PA formed by PLD may facilitate lysosomal lysis.

In conclusion, our results suggest that the phospholipid metabolism in lysosomal membranes plays an important role in the GTP γ S-dependent lysis of lysosomes, although the type of phospholipase(s) that participate(s) in the reaction is not clear. Thus, a GTP γ S-activated cytosolic G-protein may stimulate phospholipase activity, which then perturbs lysosomal membranes and results in the lysis. GTP γ S-dependent lysis may represent a unit reaction during a fusion event which involves membrane perturbation. In support of such an idea, the phase transition of membrane phospholipids from the bilayer to an H_{II} phase has been regarded as a key event in membrane fusion, and is actually induced by phospholipid metabolites

(*e.g.*, arachidonic acid and PA) (31). The assay system described in this and a previous paper may constitute a useful tool for studying the molecular mechanisms of fusion events.

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