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Disintegration of Lysosomes Mediated by GTPγS-Treated Cytosol: Possible Involvement of Phospholipases[1]

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We showed previously that cytosol treated with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) 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 ATPγS, 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 A_2. The reaction was also inhibited by phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidic acid, and phosphatidylcholine) and diacylglycerol. Among the phospholipase A_2 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 GTPγS-stimulated cytosolic phospholipase A_2 activity was highly sensitive to ONO-RS-082. These results suggest the participation of phospholipase(s), though not cytosolic phospholipase A_2, in the GTPγS-dependent lysis of lysosomes.

Key words: G-protein, GTPγS, lysis, lysosome, phospholipase A_2.

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 GTPγS-treated cytosol (*2*). The effect of GTPγS was reversed by GTP and GDP. This suggests the participation of G-protein(s) in the lysis. GTPγS-dependent lysosomal lysis involves two steps, (i) activation of the G-protein by GTPγS and (ii) ATP-dependent disintegration of lysosomes by the activated G-protein.

A number of substances are known to act as lysosome labilizers, including lipoprotein lipase, lysozyme, phospholipase A_2 (PLA_2) (*3*), and free radicals (*4, 5*). The some of activity PLA_2 is stimulated by GTPγS (*6, 7*), and the superoxide-generating NADPH oxidase system in phagocytes is stimulated by a small G-protein, rac p21 (*8*).

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 GTPγS 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

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Abbreviations: AMP-CPP, β,γ-methyleneadenosine 5′-triphosphate; AMP-PCP, α,β-methyleneadenosine 5′-triphosphate; AMP-PNP, β,γ-imidoadenosine 5′-triphosphate; ATPγS, 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; GTPγS, 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; PLA_2, phospholipase A_2; 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.

(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 μg/ml of each protease inhibitor (pepstatin A, chymostatin, antipain, and leupeptin). The homogenate was centrifuged at 1,250×*g* for 8 min and the resulting supernatant was pooled. The precipitate was homogenized again in the same buffer and then centrifuged at 550×*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×*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 GTPγS (100 μM), 350 μg protein], 1 mM ATP and 100 nM bafilomycin 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 GTPγS carried into the assay medium). After 20 min at 37°C, intact lysosomes were sedimented by centrifugation at 8,600×*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 GTPγS-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 GTPγS-treated or non-treated cytosol over broad time and temperature ranges. GTPγS-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. GTPγS-dependent disintegration of lysosomes required

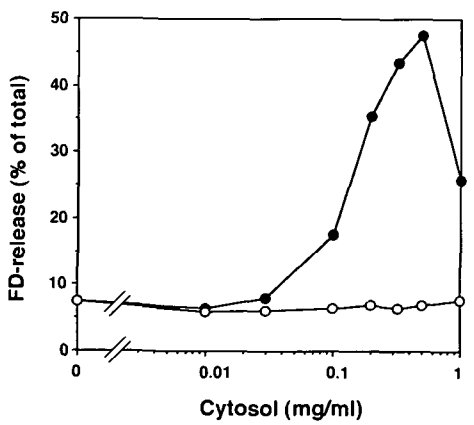

Fig. 1. **Dose-dependent effect of cytosol on lysosomal lysis.** Lysosomes isolated from rats given FD were incubated with increasing amounts of either untreated (○) or GTPγS-treated (●) cytosol for 20 min at 37°C in the presence of 1 mM ATP and 100 nM bafilomycin A_1. Intact lysosomes were sedimented 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.

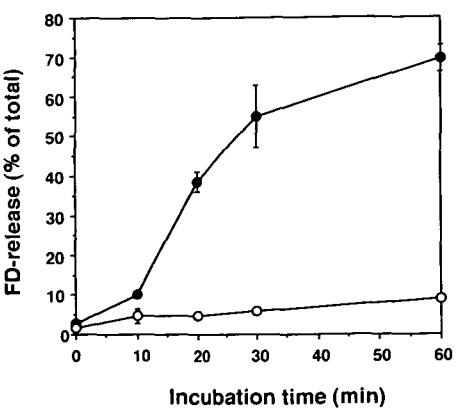

Fig. 2. **Time-course of lysosomal lysis.** Lysosomal lysis was measured over a long period of time with either untreated (○) or GTPγS-treated (●) cytosol at 37°C as described under "MATERIALS AND METHODS." Values shown are the averages and ranges of duplicates, and are representative of two experiments.

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 antibodies, nystatin and amphotericin B, both of which bind to cholesterol in membranes and restrict membrane fluidity.

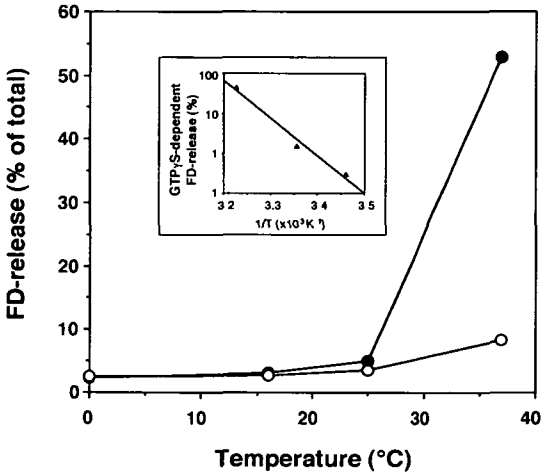

Fig. 3. **Temperature-dependence and Arrhenius plots (insert) of lysosomal lysis.** Lysosomal lysis was measured after 20 min incubation at 0, 16, 25, or 37°C with either untreated (○) or GTPγS-treated (●) cytosol as described under "MATERIALS AND METHODS." Arrhenius plots were obtained for GTPγS-dependent FD-release (FD-release by GTPγS-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 GTPγS-dependent disintegration of lysosomes.** GTPγS-dependent disintegration of lysosomes (GTPγS-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 GTPγS-treated cytosol minus that by control cytosol.

Nucleotides	GTPγS-dependent FD-release (% of control)
ATP [control]	100
dATP	109
ADP	106
ATPγS	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

ATP Requirement of GTPγS-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. ATPγS 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 Mg^{2+}. 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 bafilomycin A_1, 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 GTPγS-dependent lysosomal lysis, a series of inhibitors and known chemical

TABLE II. **Effects of ATPase inhibitors on GTPγS-dependent lysosomal lysis.** GTPγS-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	GTPγS-dependent FD-release (% of control)
None [control]	100
Sodium vanadate (1 mM)	95.6
Sodium azide (1 mM)	89.4
Oligomycin (2.5 μg/ml)	82.6
Quercetin (20 μM)	49.7

TABLE III. **Effects of various chemicals on GTPγS-dependent lysosomal lysis.** GTPγS-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	GTPγS-dependent FD-release (% of control)
None [control]	100
N-Ethylmaleimide (1 mM)	−5.6
NBD-Cl (100 μM)	2.8
DIDS (100 μM)	2.5
Diethylpyrocarbonate (1 mM)	58.5
2,3-Butanedione (10 mM)	86.8
N-Bromosuccinimide (10 μM)	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 μM)	94.8
Cytochalasin B (1 μM)	101
Amphotericin B (250 μg/ml)	27.8
Nystatin (200 μg/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 O_2^-), catalase (for H_2O_2), mannitol (for •OH), and histidine (for $^1O_2$), 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), trifluoperazine, 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 GTPγS-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 (GTPγS-independent) lysis (data not shown). Among the amphiphilic molecules, polyene antibiotics, nystatin, and amphotericin B, showed strong inhibition of the GTPγS-dependent lysis. All the molecules combine with cholesterol

TABLE IV. **Effects of phospholipase inhibitors on GTPγS-dependent lysosomal lysis.** GTPγS-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 μM.

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

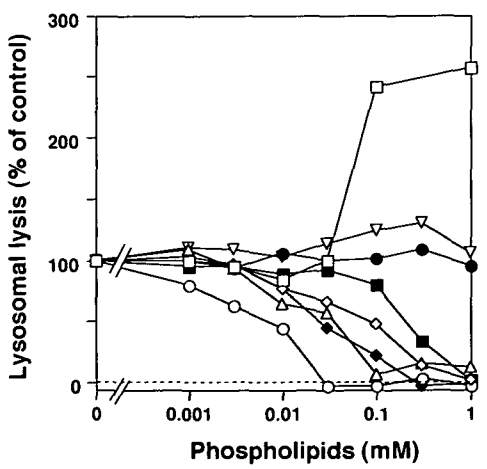

Fig. 4. **Effect of phospholipids on lysosomal integrity.** Lysosomal lysis was measured in the presence of PC (◇), PI (○), PS (◆), PA (△), lysoPC (□), DAG (■), ceramide (●), or sphingomyelin (▽), as described under "MATERIALS AND METHODS." Values shown are representative of two experiments performed with two separate lysosome preparations. The lysosomal lysis is shown as a percentage of a control that was incubated with GTPγS-treated cytosol.

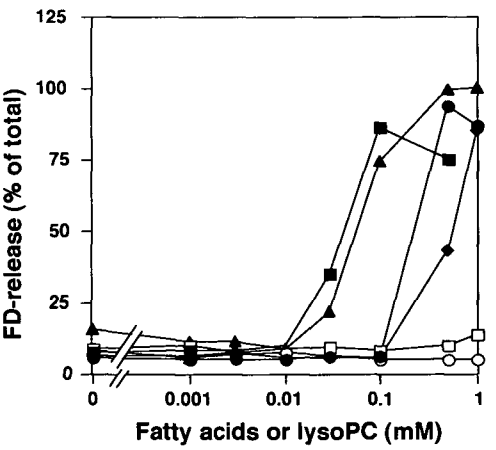

Fig. 5. **Effects of fatty acids and a lysophospholipid on lysosomal integrity.** Lysosomes were incubated with increasing amounts of either fatty acids [arachidonate (■), oleate (◆), linoleate (●), palmitate (○), and stearate (△)] or a lysophospholipid [lysophosphatidylcholine (▲)] in the presence of 350 μg/ml of non-treated cytosol, 1 mM ATP, and 100 nM bafilomycin A_1. 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.

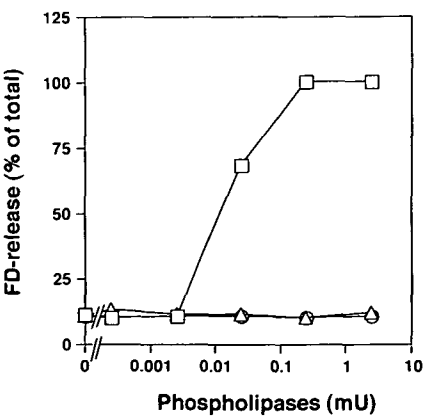

Fig. 6. **Effects of phospholipase A_2, C, and D on lysosomal integrity.** Lysosomes were examined with various amounts of phospholipases [phospholipase A_2 (□), C (△), and D (○)] for 30 min at 37°C. FD released from lysosomes was estimated as described in the legend to Fig. 1.

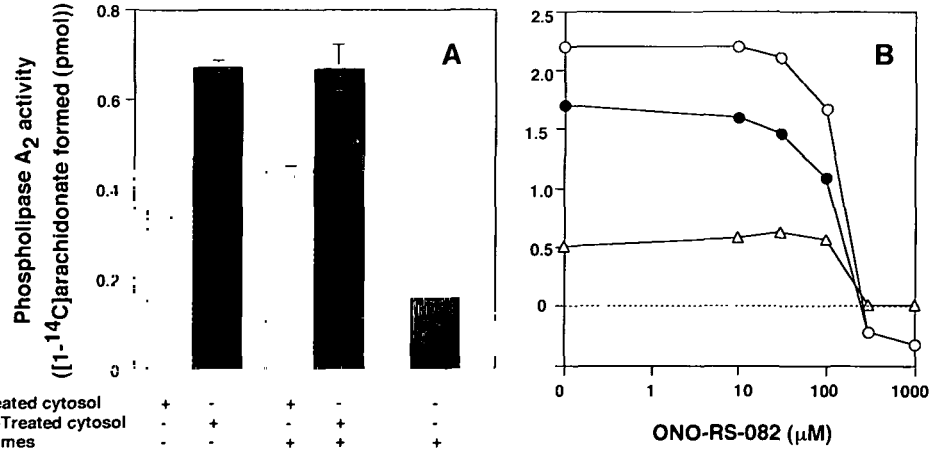

Fig. 7. **Phospholipase A_2 activities of GTPγS-treated and non-treated cytosol and lysosomes (A), and their sensitivity to ONO-RS-082 (B).** The phospholipase A_2 activities of GTPγS (300 μM)-treated and non-treated cytosol (200 μg) and lysosomes (32 μg) were assayed as described under "MATERIALS AND METHODS." In B, the phospholipase A_2 activities of GTPγS-treated cytosol (○) and non-treated cytosol (△) 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 A_2 and C (*12*), and both neomycin and spermine inhibit phospholipase C (PLC). All significantly inhibited the GTPγS-dependent lysis. But ONO-RS-082, an inhibitor of PLA_2, 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 GTPγS-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 GTPγS-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 PLA_2 are both membranolytic agents (*17, 18*). Fatty acid metabolites generated by PLA_2 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 PLA_2, 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 PLA_2 (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 PLA_2 by GTPγS—The observations described above suggest that, among various phospholipases, PLA_2 plays an important role in the GTPγS-dependent lysis of lysosomes. The GTP analoges, β,γ-imidoguanosine 5′-triphosphate and GTPγS, are potent activators of PLA_2 in permeabilized neutrophils and HL60 cells, and it was reported that pretreatment of homogenates of HL-60 granulocytes with specific antibodies against cytosolic PLA_2 attenuated the basal as well as stimulated arachidonate release (*21*). The presence of PLA_2 activity in lysosomes has also been reported (*22*). Therefore, cytosolic or lysosomal PLA_2 might well be regulated by G proteins. In fact, in our experiments, GTPγS significantly stimulated the activity of cytosolic PLA_2, but not that of lysosomal PLA_2 (Fig. 7A). However, the GTPγS-stimulated PLA_2 was totally inhibited by ONO-RS-082, a PLA_2 inhibitor which does *not* inhibit GTPγS-stimulated lysis (Fig. 7B). These results suggest that cytosolic PLA_2 does not participate in the lysis.

DISCUSSION

In this study, we showed that disintegration of rat liver lysosomes induced by GTPγS-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 GTPγS-dependent lysis have widely varying pharmacological properties, but share cationic amphiphilic features and inhibit phospholipases (A_2, 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 GTPγS-dependent lysis.

It is unlikely that the energy for the GTPγS-dependent lysis of lysosomes is provided by ATP, because ADP and a nonhydrolyzable analog of ATP, ATPγS, 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 (PLA_2, PLC, and PLD) are activated by GTPγS 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, PLA_2 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 GTPγS (*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.*, lysophospholipids 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 PLA_2, but not PLC or PLD, disintegrated lysosomes. And unsaturated fatty acids and lysoPC, products of the PLA_2 action, also induced the lysis. Moreover, we found that PLA_2 activity in rat liver cytosol was activated significantly on treatment with GTPγS. However, both the basal and GTPγS-stimulated cytosolic PLA_2 activities were inhibited by ONO-RS-082, which had no detectable effect on the GTPγS-dependent lysis. This suggests that cytosolic PLA_2 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 GTPγS-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 GTPγS-dependent lysis of lysosomes, although the type of phospholipase(s) that participate(s) in the reaction is not clear. Thus, a GTPγS-activated cytosolic G-protein may stimulate phospholipase activity, which then perturbs lysosomal membranes and results in the lysis. GTPγS-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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