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ATP-Mediated Activation of Ca^{2+}-Independent Phospholipase A_2 in Secretory Granular Membranes from Rat Parotid Gland[1]

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We characterized the Ca^{2+}-independent, membrane-associated phospholipase A_2 (PLA_2) from rat parotid secretory granules. Among four phosphatidylcholine species with different fatty acyl (palmitoyl, oleoyl, linoleoyl, and arachidonoyl) groups at the *sn*-2 position, 2-arachidonoyl-phosphatidylcholine was the preferred substrate. Such specificity was also apparent even when 2-arachidonoyl-phosphatidylcholine coexisted with another species. The various well-documented inhibitors of PLA_2s, bromo*enol* lactone, arachidonyl trifluoromethyl ketone, methyl arachidonyl fluorophosphate, and diisopropyl fluorophosphate, did not inhibit granular PLA_2 activity. The granular PLA_2 was activated markedly by ATP, and to a lesser extent by GTP and ATPγS. GTP also partially suppressed the ATP-mediated activation. UTP, CTP, GTPγS, and the hydrolyzed products of ATP and GTP showed little activation of the enzyme. Neither addition of K-252a nor depletion of Mg^{2+} affected ATP-mediated activation. Although this enzyme was located in the granular membranes, the granular soluble contents or BSA were required for the full activity and full ATP-mediated activation. These results suggested that the PLA_2 located in granular membranes may participate in the liberation of arachidonic acid in parotid cells and be regulated through a mechanism mediated by ATP.

Key words: arachidonic acid, ATP, Ca^{2+}-independent phospholipase A_2, rat parotid gland, secretory granular membranes.

The activation of intracellular phospholipase A_2 (PLA_2) has important roles in the regulation of cellular function (*1-3*). In secretory systems, the functional relationship between PLA_2 and the exocytotic pathway has been discussed (*4-6*). Morgan and Burgoyne (*7*) reported that the release of catecholamine from permeabilized chromaffin cells was enhanced by a protein named Exo I, which shares sequence homology with PLA_2 (*8*). Murakami *et al.* demonstrated the suppression of histamine release from mast cells by group II-PLA_2 specific inhibitors (*9*). Furthermore, based upon studies using a cell-free system, it has been proposed that PLA_2 is involved in the intra-Golgi transport (*10*) and/or the interaction between secretory granules and plasma membranes (*11, 12*).

Previously, we reported a cell-free interaction system consisting of isolated secretory granules and plasma membranes from the rat parotid gland (*13*). In this system, the plasma membranes evoke amylase release from isolated secretory granules without any additional factors. Furthermore, liposomes mimicked the effect of plasma membranes (*14*). We therefore proposed that the ability for exocytotic interaction is inherent in secretory granules and plasma membranes. In addition, we also demonstrated the presence of PLA_2 in secretory granular membranes from the rat parotid gland (*15*), suggesting that it plays a regulatory role in exocytosis. However, little is known about this enzyme.

In this study, we characterized the PLA_2 activity localized in secretory granular membranes from the rat parotid gland and showed its acyl chain specificity and activation mediated by ATP.

MATERIALS AND METHODS

Materials—Male Wistar rats (9-12 weeks old) were maintained on Oriental MF solid chow (Oriental Yeast, Tokyo) and water *ad libitum*. After fasting overnight, rats were killed by bleeding under light diethyl ether anesthesia. Immediately, the parotid glands were isolated and trimmed of connective tissue.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (16:0/16:0-GPC) and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0/18:2-GPC) were purchased from Sigma Chemi-

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Abbreviations: $AACOCF_3$, arachidonyl trifluoromethyl ketone; ATPγS, adenosine 5′-[γ-thio]triphosphate; BEL, bromo*enol* lactone [(*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one]; DFP, diisopropyl fluorophosphate; GDPβS, guanosine 5′-[β-thio]diphosphate; GTPγS, guanosine 5′-[γ-thio]triphosphate; 16:0/16:0-GPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 16:0/18:1-GPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 16:0/18:2-GPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; 16:0/20:4-GPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; MAFP, methyl arachidonyl fluorophosphate; PLA_2, phospholipase A_2.

cal (St. Louis, MO) and Serdary Research Laboratories (London), respectively. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0/18:1-GPC) and 1-pal-mitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (16:0/20:4-GPC) were obtained from Avanti Polar Lipids (Birmingham, AL). 1-Palmitoyl-2-[^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine (1.85-2.22 TBq/mmol), 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (2.13 GBq/mmol), 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phospho-choline (1.85 GBq/mmol), and 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (1.85-2.22 GBq/mmol) were purchased from DuPont/New England Nuclear Research Products (Boston, MA). They were diluted, if necessary, with the same unlabeled phosphatidylcholine (PC) species to give a specific radioactivity suitable for phospholipase assay (40,000-80,000 dpm/nmol). All labeled PCs were purified by TLC with chloroform/methanol/water (65:25:4, v/v) and their radiochemical purity was more than 98% according to TLC.

ADP, AMP, GDP, GMP, GDPβS, and GMP were purchased from Sigma and, ATPγS, GTP, GTPγS, UTP, and CTP were from Boehringer Mannheim (Indianapolis, IN). ATP was purchased from both Sigma and Boehringer Mannheim and its purity was more than 99%. The other nucleotides were of the highest grade available. Sodium pyrophosphate was from Nacalai Tesque (Kyoto). All nucleotides and their derivatives were dissolved and neutralized (at pH 7.0) using appropriate buffers. K-252a was purchased from Calbiochem-Novabiochem (La Jolla, CA) and was reconstituted with 1 ml of dimethyl sulfoxide (DMSO, Nacalai Tesque). Bromo*enol* lactone[(*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one] (BEL) and arachidonyl trifluoromethyl ketone (AACOCF$_3$) purchased from BIOMOL Research Lab. (Plymouth Meeting, PA) were freshly dissolved in DMSO and used for PLA$_2$ assay. Methyl arachidonyl fluorophosphate (MAFP) and diisopropyl fluorophosphate (DFP) were purchased from Cayman Chemical (Ann Arbor, MI) and Wako Pure Chemical Ind. (Osaka), respectively. Two millimolar MAFP and 1 M DFP solutions (in DMSO) were stored at −30 and 4°C, respectively, as stock solutions. BSA (fraction V) was purchased from Sigma.

Preparation of Secretory Granules and Their Membrane Fraction—Secretory granules were purified from male Wistar rat parotid glands by differential and Percoll gradient centrifugations as described previously (*15*).

The purified granules were lyzed by freezing and thawing (two or three repetitions) in a hypotonic buffer (2 mM Mops-NaOH, pH 7.0). Granular membranes were collected by centrifugation at 150,000×*g* for 90 min. The supernatant was recentrifuged at 300,000×*g* for 60 min to remove membranous contaminants from the soluble contents. The purified membranes were washed twice with the same hypotonic buffer, resuspended in 20 mM Mops-NaOH (pH 7.0) containing 0.3 M sucrose, and stored at −80°C.

Solubilization of Granular Membranes—Granular membranes were suspended in 20 mM Mops-NaOH (pH 7.0) containing 0.3 M sucrose in the presence of 0.5% Triton X-100 to yield 3 mg protein/ml. Mixtures were gently stirred at 4°C for 2 h, then centrifuged at 105,000×*g* for 2 h at 4°C (Optima TLX: Beckman Instruments, Palo Alto, CA). The supernatant (solubilized fraction) was directly used for enzyme assay.

For the control, granules were treated in the same way except that solubilization with Triton X-100 was omitted. After centrifugation, the PLA$_2$ remained in the resulting pellet (control pellet), and was hardly detected in the supernatant.

Recoveries of protein by this procedure were 62% (control) and 66% (Triton X-100 treatment).

PLA$_2$ Assay—PLA$_2$ activity was measured essentially as described previously (*15*). Briefly, the standard incubation mixture contained, in 0.1 ml: 0.1 mg of granular protein, 180 μM 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine, 10 mM EDTA or EGTA, 0.05% Triton X-100, and 100 mM Mops-NaOH (pH 7.0). Labeled substrate was added as a liposomal suspension (1.8 mM). The reactions were initiated by the addition of enzyme protein, carried out at 37°C for 60 min and terminated with 0.3 ml of chloroform/methanol (1 : 2, v/v). The products were extracted according to the method of Bligh and Dyer (*16*) and separated on a 0.2 M boric acid-impregnated Silica Gel 60 plate (Merck, Darmstadt) with chloroform/acetone (9 : 1, v/v). The radioactivity of separated free fatty acid was measured in an Aloka LSC-900 liquid scintillation counter as described previously (*17*). The values for nonenzymatic hydrolysis in the complete incubation mixture without enzyme protein were 0.01-0.09% of total radioactivity added. The values for net hydrolysis were obtained by subtraction of the values for non-enzymatic hydrolysis. The reaction was dependent on the amount of protein up to 0.3 mg (with the whole granular fraction) or to 0.015 mg (with the granular membrane fraction).

Inhibition by Various PLA$_2$ Inhibitors—The PLA$_2$ inhibitor (*e.g.* BEL, MAFP, or DFP) was injected (1 μl) in a buffer consisting of 100 mM EGTA, 0.05% Triton X-100, and 20 mM Mops-NaOH, pH 7.0. After preincubation with the inhibitor and secretory granules for 5 min at 37°C, labeled substrate was added followed by incubation at 37°C for 60 min and the reaction was terminated with 0.3 ml of chloroform/methanol (1 : 2, v/v). In the case of AACOCF$_3$, the inhibitor was injected (1 μl) in a buffer consisting of 180 μM labeled substrate, 100 mM EGTA, 0.05% Triton X-100, and 20 mM Mops-NaOH, pH 7.0, and the reaction was started by adding secretory granules. The inhibitory effects of individual inhibitors on myocardial Ca^{2+}-independent PLA$_2$ were confirmed using rat myocardial cytosol according to Hazen *et al.* (*18, 19*).

Biochemical Analyses—The protein concentration was determined by the method of Lowry *et al.* (*20*) using bovine serum albumin as the standard. The concentration of PC was determined based upon the method of Bartlett (*21*) as modified by Marinetti (*22*).

RESULTS

Acyl Chain Specificity of PLA$_2$ from Rat Parotid Secretory Granules—The substrate specificity of granular PLA$_2$ for the acyl chain of PC was examined by comparing the fatty acids released from the four PC species shown in Fig. 1. Our analysis of the positional distribution of fatty acids in PC (*23, 24*) suggested that these species are predominant in PC from the rat parotid gland. All PC species used in this assay saturated at a similar concentration (about 100 μM). The activity for arachidonoyl-containing PC was the highest among those for four species. However, the preference for 16:0/20:4-GPC was only 1.8, 2.9, and 5.6-fold

with 16:0/18:1-, 16:0/18:2-, and 16:0/16:0-GPC, respectively, which seems to be relatively low. Such substrate specificity was also observed even when 16:0/20:4-GPC was mixed with another species in the incubation mixture (Table I). The release of arachidonic acid from mixed substrates was always greater than that of the other fatty acids. The ratios of arachidonic acid released from mixed substrates to the other fatty acid released (7.9, to 16:0; 1.5, to 18:1; 2.9, to 18:2) were similar to those measured using a single substrate. These results indicate that the selectivity of this enzyme may be independent of alterations in the physical properties and interfacial characteristics of the substrate.

Effects of Various Inhibitors on Granular PLA_2 Activity—The effects of various PLA_2 inhibitors on granular PLA_2 activity were investigated to determine the properties of this enzyme (Figs. 2 and 3). Well-documented intracellular Ca^{2+}-independent PLA_2s (*e.g.*, Ca^{2+}-independent PLA_2s from the myocardium and $P388D_1$ macrophage-like cells) have been reported to be inhibited by

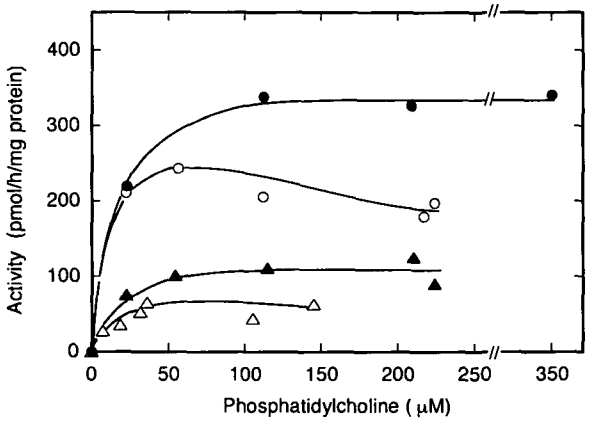

Fig. 1. **Fatty acyl chain specificity of phospholipase A_2 in rat parotid secretory granules.** The PLA_2 activity in secretory granules was assayed using four species (△, 1,2-dipalmitoyl-; ○, 1-palmitoyl-2-oleoyl-; ▲, 1-palmitoyl-2-linoleoyl-; ●, 1-palmitoyl-2-arachidonoyl-) of phosphatidylcholine in 0.05% Triton X-100, 10 mM EDTA, and 100 mM Mops-NaOH (pH 7.0). Each substrate was added to the assay mixture as a liposomal suspension prepared from a single PC species. The specific activity in the presence of inhibitor was regarded as 100%. The values are means of duplicate determinations.

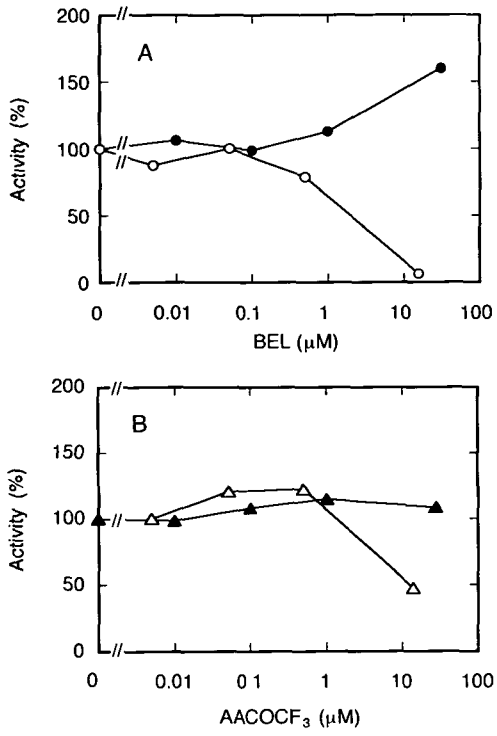

Fig. 2. **Effects of the two PLA_2 inhibitors on the phospholipase A_2 activities in rat parotid secretory granules and in rat myocardial cytosol.** A: Effect of BEL, secretory granules (●) were preincubated at 37°C for 5 min with 0.05% Triton X-100, 10 mM EGTA, 100 mM Mops-NaOH (pH 7.0), and 1% DMSO in the absence or presence of the indicated concentrations of BEL. The reactions were initiated by addition of labeled substrate and performed at 37°C for 60 min. Myocardial cytosol (○) was preincubated at 20°C for 5 min with 4 mM EGTA, 5% glycerol, 100 mM Tris-HCl (pH 7.0), and 0.5% DMSO in the absence or presence of the indicated concentrations of BEL according to Hazen *et al.* (*19*). Labeled substrate was added followed by preincubation at 37°C for 5 min. B: Effect of $AACOCF_3$, the reactions were initiated by addition of SG (▲) or myocardial cytosol (△) after preincubation of labeled substrate with the indicated concentrations of $AACOCF_3$ for 5 min at 37°C (secretory granules) or 20°C (myocardial cytosol) in the assay mixtures described in "A." The incubation was performed at 37°C for 60 min (secretory granules) or 5 min (cytosol). Activity is expressed as a percentage of the specific activity in the absence of inhibitors. The values are means of two or more independent assays.

TABLE I. **Fatty acyl chain selectivity of parotid granular phospholipase A_2 in the mixed substrate.**

Phosphatidylcholines	Release of[a]		Ratio[b]
	[^{14}C]20:4[c]	Other labeled FFA	
Exp. 1			
50 μM 16:0/[^{14}C]20:4[d] +50 μM 16:0/[^{3}H]16:0	143±4	18±7	7.9
Exp. 2			
50 μM 16:0/[^{14}C]20:4 +50 μM 16:0/18:1	110±4		1.5
50 μM 16:0/20:4 +50 μM 16:0/[^{14}C]18:1		74±5	
Exp. 3			
50 μM 16:0/[^{14}C]20:4 +50 μM 16:0/18:2	113±4		2.9
50 μM 16:0/20:4 +50 μM 16:0/[^{14}C]18:2		39±4	

Secretory granules were incubated with mixed phosphatidylcholine substrate in the presence of 0.05% Triton X-100, 10 mM EDTA and 100 mM Mops-NaOH (pH 7.0). Hydrolysis of individual fatty acyl moieties in the mixed substrate was measured independently, using single species-labeled substrate. Only for 16:0/16:0 and 16:0/20:4 was a double-labeled substrate used. All labeled fatty acyl chains occupied the *sn*-2 position. Values are means±SE of triplicate determinations. [a]pmol/h/mg protein. [b]Ratio of the release of [^{14}C]arachidonic acid to that of the other labeled free fatty acid in the individual experiments. [c]Fatty acyl moieties are designated by the number of carbon atoms: the number of unsaturations. [d]Molecular species of phosphatidylcholine are designated by the fatty acyl chain at *sn*-1 position/the fatty acyl chain at *sn*-2 position.

BEL, a mechanism-based inhibitor (*2, 3, 19, 25*). Actually, in the present study, preincubation of BEL with rat myocardial cytosol resulted in concentration-dependent inhibition of its Ca^{2+}-independent PLA_2 activity (Fig. 2A). However, BEL showed little effect on the granular PLA_2 activity at concentrations up to 1 μM. A higher concentration (30 μM) of BEL rather activated granular PLA_2 (Fig. 2A). $AACOCF_3$, a selective inhibitor of 85-kDa cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), was reported to inhibit the Ca^{2+}-independent PLA_2 from $P388D_1$ cells (*25*). In addition, we observed partial inhibition of Ca^{2+}-independent PLA_2 activity of myocardial cytosol at high concentration (15 μM, Fig. 2B). On the other hand, the granular PLA_2 activity was not affected by $AACOCF_3$ even at 30 μM (Fig. 2B).

Some PLA_2s including Ca^{2+}-independent PLA_2 have the active site Ser in their catalytic domain (*26, 27*). In the present study, the two serine hydrolase inhibitors, methyl arachidonyl fluorophosphate, and diisopropyl fluorophosphate, inhibited Ca^{2+}-independent PLA_2 activity of myocardial cytosol (Fig. 3). However, these inhibitors did not affect granular PLA_2 activity. These observations suggest that the granular enzyme is different from the other well-documented PLA_2s.

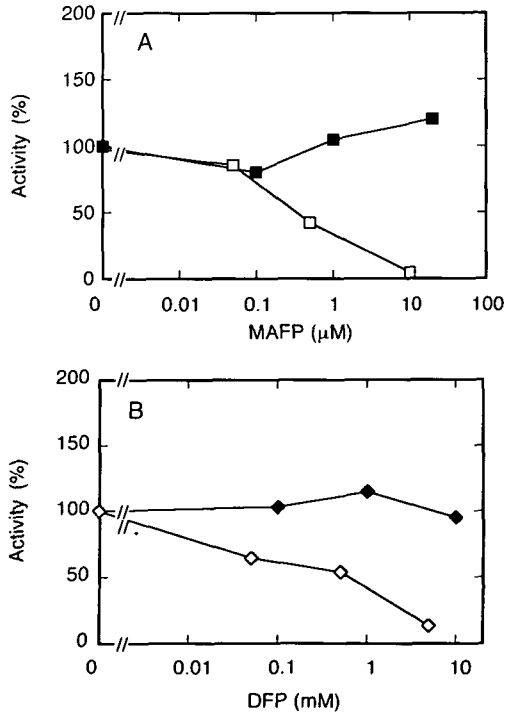

Fig. 3. **Effects of serine esterase inhibitors on the phospholipase A_2 activities in rat parotid secretory granules and in rat myocardial cytosol.** Secretory granules (■, ◆) were preincubated at 37°C for 5 min with 0.05% Triton X-100, 10 mM EGTA, 100 mM Mops-NaOH (pH 7.0), and 1% DMSO in the absence or presence of the indicated concentrations of inhibitor (MAFP, ■; or DFP, ◆). The reactions were initiated by addition of labeled substrate and performed at 37°C for 60 min. Myocardial cytosol (□, ◇) was preincubated at 20°C for 5 min with 4 mM EGTA, 5% glycerol, 100 mM Tris-HCl (pH 7.0), and 0.5% DMSO in the absence or presence of the indicated concentrations of inhibitor (MAFP, □; or DFP, ◇). Labeled substrate was added, followed by incubation at 37°C for 5 min. Activity is expressed as a percentage of the specific activity in the absence of inhibitors. The values are means of two independent assays.

Effects of Various Nucleotides on Granular PLA_2 Activity—To identify a modulator of the granular PLA_2, the effects of four naturally occurring nucleotide triphosphates and their derivatives on the enzyme activity were investigated. As shown in Fig. 4, ATP markedly increased granular PLA_2 activity. This effect was observed at ATP concentrations above 1 mM and did not reach the maximum even at 100 mM. GTP and ATPγS also activated the PLA_2 in a concentration-dependent manner, whereas UTP and CTP had little effect. Thus, the alteration was restricted to purine nucleotides. The most potent activator was ATP, although its maximal effect was manifested at a very high concentration (more than 100 mM). On the contrary, at a lower concentration (<10 mM), GTP-mediated activation was greater than that by ATP. At all concentrations, ATPγS had a lower effect than the others, and GTPγS had none. Thus, the hydrolyzable form was more efficient than the nonhydrolyzable form. However, none of the hydrolysis products of ATP and GTP significantly activated the PLA_2 (Fig. 5). The ATP-mediated activation disappeared after decomposing ATP at high temperature (600°C for 1–2 h, data not shown). These results indicated that the likelihood of the effector being a hydrolysis product or an inorganic contaminant in ATP and GTP can be excluded. BEL did not inhibit the activity increased by ATP (data not shown). Figure 6 shows that the coexistence of GTP with ATP rather suppressed the ATP-mediated activation, suggesting that the two purine nucleotide triphosphates activate the PLA_2 through the same mechanism. It is possible that GTP acts as a partial antagonist of ATP.

Table II shows the effects of divalent cations on ATP-mediated activation. Replacing EGTA with EDTA had no effect on the ATP- and GTP-mediated activation of PLA_2 activity. This suggested that the effect is independent of Mg^{2+}. This independence of Mg^{2+} and the lower potency of GTP compared with ATP may exclude the notion that conventional G-protein mediates the activation (*28–30*).

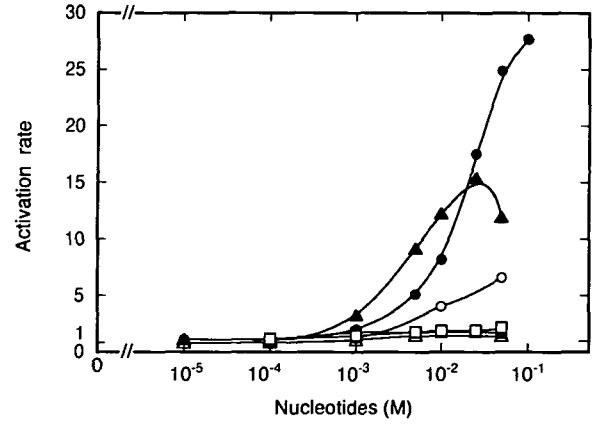

Fig. 4. **Nucleotide specificity in activation of phospholipase A_2 in rat parotid secretory granules.** Secretory granules were incubated with 180 μM labeled substrate in 0.05% Triton X-100, 10 mM EGTA, and 100 mM Mops-NaOH (pH 7.0) containing various nucleotides (●, ATP; ○, ATPγS; ▲, GTP; △, GTPγS; ■, UTP; □, CTP). The activation rate is expressed as the ratio of the specific activity in the presence of individual nucleotides and their derivatives to that in their absence. The values are means of duplicate determinations.

On the other hand, Ca^{2+} perturbed the nucleotide-mediated effect. $CaCl_2$ markedly suppressed the ATP- and GTP-mediated activation.

Figure 7 shows the effects of K-252a, a nonspecific and potent inhibitor of various protein kinases (*31*). In this experiment, the concentration of ATP was lowered as much as possible since the mechanism of inhibition by K-252a was reported to be competition with ATP (*31*). Nevertheless, K-252a affected neither PLA_2 activity (data not shown) nor its ATP-mediated activation (Fig. 7) at concentrations up to 2 μM, which is 100-1,000-fold higher than the K_i values of various protein kinases (*31*).

Effects of Granular Soluble Contents on ATP-Stimulatable PLA_2 Activity—Previously, we observed that the activity of granular PLA_2 was totally lost when the membranes were separated from the granular soluble contents (*15*). Therefore, we further examined the effects of the soluble contents on PLA_2 activity and ATP-mediated activation. When membranes were separated from the granular soluble contents, almost all PLA_2 activity was detected in the granular membrane fraction (SG-M) regardless of the presence or absence of ATP (Table III). Therefore, ATP-stimulatable PLA_2 seems to be strictly located in granular membranes (not in the granular soluble fraction, SG-S), and the activity in the whole granules should reflect that associated with their membranes. However, the total PLA_2 activity recovered in SG-M was much lower than that in whole granules, despite good recovery of protein in SG-S and SG-M (92.0 and 3.1%, respectively). These results indicated that isolation of the membranes from whole granules decreased the activity of the membrane-associated PLA_2. Furthermore, the magnitude of ATP-mediated activation, using SG-M as an enzyme source, was also less than that using whole granules (Table III). When SG-S was returned to the incubation mixture, the total PLA_2 activity and the magnitude of

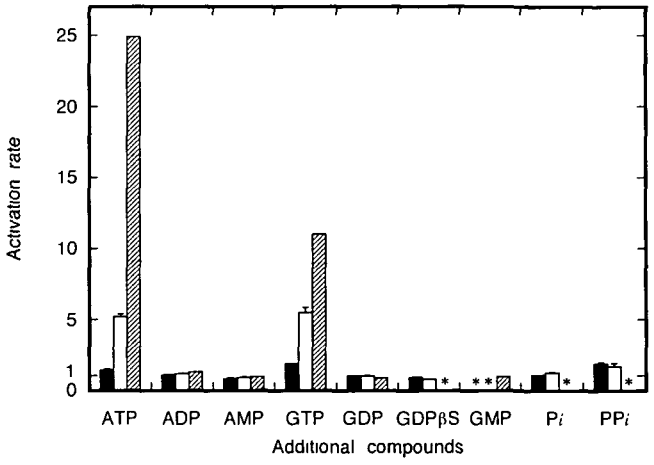

Fig. 5. **Effects of ATP and GTP derivatives on phospholipase A_2 activity in rat parotid secretory granules.** Secretory granules were incubated with 200 μM labeled substrate in the presence of 0.05% Triton X-100, 10 mM EGTA, and 100 mM Mops-NaOH (pH 7.0) under various conditions. The activation rate is expressed as the ratio of the specific activity in the presence of individual ATP- and GTP-derivatives to that in their absence. Closed, open, and hatched bars indicate 1, 8, and 50 mM nucleotide-derivatives, respectively. The values are means. SE ($n=3$) is indicated where appropriate. The other values are means of duplicate determinations. Asterisks indicate that values were not determined.

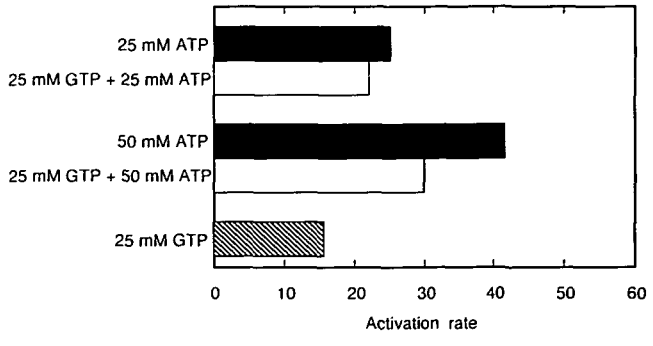

Fig. 6. **Effects of combinations of ATP and GTP on phospholipase A_2 activity in rat parotid secretory granules.** Secretory granules were incubated with 180 μM labeled substrate in 0.05% Triton X-100, 10 mM EGTA, and 100 mM Mops-NaOH (pH 7.0) containing ATP and/or GTP. The activation rate is expressed as the ratio of the specific activity in the presence of nucleotides to that in their absence. The values are means of duplicate determinations.

TABLE II. **Effects of divalent cations on the ATP- and GTP-mediated activation of phospholipase A_2 in rat parotid secretory granules.**

Addition	Activation rate[a]		
	10 mM EDTA	10 mM EGTA	5 mM $CaCl_2$
None	1 (399)[b]	1 (380)	1 (329)
ATP	5.12	5.11	1.83
GTP	7.31	9.18	1.66

Secretory granules were incubated with 180 μM labeled substrate under various conditions. The values are means of two to five determinations. [a]The ratio of the specific activity in the presence of an additional compound to that in its absence under the given divalent cation conditions. [b]Values in parentheses are the specific activities (pmol/h/mg protein) of PLA_2 in the absence of an additional compound.

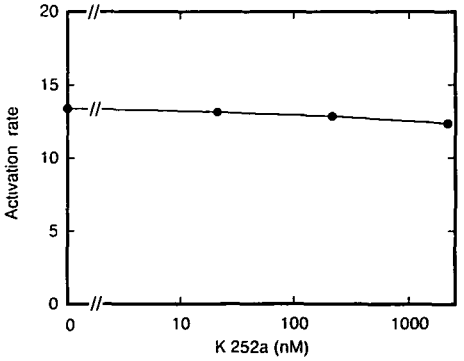

Fig. 7. **Effects of the protein kinase inhibitor K-252a on ATP-mediated activation of phospholipase A_2 in rat parotid secretory granules.** Secretory granules were preincubated at 37°C for 10 min with 0.05% Triton X-100, 10 mM EGTA, 100 mM Mops-NaOH (pH 7.0), and 1% DMSO in the absence or presence of the indicated concentrations of K-252a. Then ATP was added to a final concentration of 10 mM. Five minutes after addition of ATP or water, 190 μM labeled substrate was added, followed by incubation at 37°C for 60 min. In the control, SG received the same treatment in the absence of ATP. The activation rate is expressed as the ratio of the specific activity in the presence of ATP to that in its absence. The values are means of duplicate determinations.

TABLE III. **Effect of the granular soluble fraction on the membrane-associated phospholipase A_2 activity in rat parotid secretory granules.**

Fraction	Specific activity (nmol/h/mg protein)		Total activity(nmol/h)		Activation rate[a]
	None	ATP	None	ATP	
Whole granules	0.9±0.1	13.5±0.7	61.9±3.4	926.4±50.9	15.0
Granular soluble fraction (SG-S)	<0.1	0.2±0.0	<0.1	12.9±0.7	n.d.[b]
Granular membrane fraction (SG-M)	17.1±1.4	121.7±30.1	35.2±3.0	256.9±63.5	7.1
SG-M+SG-S	22.0±1.2	303.3±20.6	48.5±4.2	640.0±43.4	14.0

The PLA_2 activity was assayed using whole granules (0.1 mg), the granular soluble fraction (0.1 mg), the granular membrane fraction (0.004 mg), or the granular membrane fraction (0.004 mg) combined with the granular soluble fraction (0.1 mg) as an enzyme source. The amount of granular membrane fraction (0.004 mg of protein) used for the enzyme assay is theoretically equal to the amount of membrane protein in the whole granules used. The values are means±SE of three independent assays. [a]Ratio of the specific activity in the presence of ATP (40 mM) to that in its absence. [b]Not determined.

TABLE IV. **Solubilization of phospholipase A_2 with Triton X-100.**

	Activity recovered (%)	Specific activity[a] (nmol/h/mg protein)	Activation rate[b]
Control pellet	100[c]	10.5	11.1
Solubilized fraction	31.1	4.6	9.5

The PLA_2 activity was assayed using 0.005 mg of protein, 180 μM labeled substrate, 0.05% Triton X-100, 10 mM EGTA, and 100 mM Mops-NaOH (pH 7.0) in the presence or absence of 50 mM ATP. The solubilized fraction was used without dialysis for enzyme assay and final concentration of Triton X-100 in the assay mixture was adjusted to 0.05%. The values are means of duplicate determinations. [a]Specific activity in the absence of ATP. [b]Ratio of the specific activity in the presence of ATP to that in its absence. [c] Total activity in the control pellet was regarded as 100%.

ATP-mediated activation in SG-M were recovered to 80 and 93% of those before separation, respectively (Table III). These results indicated that the granular soluble contents are required for the full activity and activation of the membrane-associated PLA_2 *in vitro*.

On the other hand, the granular soluble fraction denatured by heating (95°C for 5–10 min) did not affect the total activity but increased ATP-mediated activation (data not shown). BSA (fraction V) or cytosol from the rat parotid gland mimicked the effects of SG-S on the total activity and the ATP-mediated activation (data not shown). These observations indicated that the effects are not strictly specific to granular soluble contents. In our preliminary experiment, the addition of free fatty acid to the substrate suspension reduced the granular PLA_2 activity (data not shown). Analogous to BSA, the soluble proteins in secretory granules and the cytosol may contribute to removal of the products from the enzyme protein.

Solubilization of ATP-Stimulatable PLA_2 with Triton X-100—The properties of membrane-bound enzymes may be changed by solubilization and/or further purification. Therefore, we examined the effects of solubilization on ATP-mediated activation of PLA_2. Triton X-100 solubilized about 50% of granular membrane protein (data not shown). More than 30% of the original PLA_2 activity was recovered in the supernatant (Table IV). Although specific activity in the solubilized fraction was reduced to half of that in the control pellets, ATP increased the activity in the solubilized fraction as well as in the control pellets.

DISCUSSION

We have reported herein the unique properties of Ca^{2+}-independent PLA_2 in rat parotid secretory granular membranes.

One class of higher molecular weight (70–100 kDa) cytosolic phospholipase A_2s ($cPLA_2$) has been considered as an intracellular messenger, since it is fully activated at a low level of Ca^{2+} and selectively hydrolyzes the arachidonoyl chain of phospholipids (*1, 32–35*). The preference of $cPLA_2$ for the arachidonoyl chain is over 10-fold higher than that for the other unsaturated acyl chains (*33, 34*). In our mixing experiment (Table I), the granular PLA_2 also showed some preference for the arachidonoyl chain, especially compared with the palmitoyl chain (8-fold). However, the preference of granular PLA_2 for the arachidonoyl chain was only 1.5- and 3-fold more than for the oleoyl and linoleoyl chains, respectively. The granular PLA_2 does not seem to discriminate among unsaturated fatty acyl moieties as strictly as $cPLA_2$. Thus, it appears that the granular PLA_2 is different from $cPLA_2$ not only in its Ca^{2+}-independence but also in its acyl chain specificity. Furthermore, immunoblotting analysis demonstrated that polyclonal antibody (IgG 1_κ) against $cPLA_2$ did not specifically recognized any of the granular membrane proteins resolved on SDS-polyacrylamide gel electrophoresis (8%) (unpublished data). The $cPLA_2$ selective inhibitor, $AACOCF_3$, hardly inhibited activity of the granular PLA_2. These observations suggest that granular PLA_2 can be classified into a different group from $cPLA_2$.

Over the last decade, other PLA_2s have been identified which do not depend on Ca^{2+} for activity (*2, 3, 18, 19, 27, 35–41*). The two Ca^{2+}-independent PLA_2s from the myocardium and the macrophage-like cell line $P388D_1$ have been most extensively studied by Gross *et al.* (*18, 19, 36, 37*) and Dennis *et al.* (*25, 27, 40*), respectively. These enzymes differ in their molecular weight, substrate specificity and detergent sensitivity. However, they are unique among known PLA_2s in that they are both activated by ATP and inhibited by the mechanism-based inhibitor BEL. In addition, they were inhibited by a serine esterase inhibitor (*27*, Fig. 3), which indicates that they have the Ser in their active site. In the present study, we demonstrated that the Ca^{2+}-independent PLA_2 associated with secretory granular membranes was also activated by ATP. However, unlike myocardial and $P388D_1$ PLA_2s, it showed little inhibition by BEL regardless of the presence or absence of ATP. In addition, two serine esterase inhibitors also had no effect. The granular Ca^{2+}-independent PLA_2 seems to be different from the myocardial and $P388D_1$ PLA_2s.

In comparison with the myocardial and $P388D_1$ PLA_2s, the granular PLA_2 required an extremely high concentra-

tion of ATP for maximal activation. Then, we investigated the effects of possible contaminants and natural decomposition products described in the analytical data sheet of this ATP preparation (*i.e.*, inorganic contaminant and hydrolyzed products of ATP) on the granular PLA_2 activity. As shown in "RESULTS," none of these products activated the granular PLA_2. The high requirement of ATP can probably be explained by the fact that the granular PLA_2 was not purified. It is possible that the granular PLA_2 competes with other enzymes in the granules [*e.g.*, protein kinase (*42*) and H^+-ATPase (*43*)] for ATP in the assay system. Alternatively, the concentration for maximal activation *in vitro* seemed to be partially dependent on the assay conditions such as the species of substrate used (*38*) and the concentration of Triton X-100 (*40*). Setting aside the question of the high requirement of ATP, we clearly demonstrated that the nucleotide-mediated activation was restricted to purine nucleotide triphosphates. Although the enzyme has not yet been purified, its solubilized activity was also increased by ATP. Furthermore, in our preliminary experiment, a significant amount of the solubilized activity bound to the ATP-agarose matrix (unpublished data). From these observations, we concluded that ATP itself is involved in the activation of this enzyme, at least *in vitro*.

The present results are not sufficient for determination of the mechanism of ATP-mediated activation of this enzyme. From our findings, however, we can exclude the three possibilities listed below. Firstly, the involvement of conventional G proteins in the ATP-mediated activation (*28–30*) can be excluded as ATP was more potent than GTP, and Mg^{2+} was a nonobligatory component of ATP-mediated activation (Fig. 3 and Table II). Secondly, K-252a failed to inhibit the effects of ATP (Fig. 6), suggesting that a classic phosphorylation reaction was not required (*31*). The observation that a nonhydrolyzable analogue of ATP (ATPγS) activated PLA_2 (Fig. 3) also supports this suggestion. Lastly, the fact that ATP activated both the solubilized and the membrane-bound PLA_2 (Table IV) indicates that the activation of granular PLA_2 by ATP was not the result of changes in the lipid environment, although it is well known that the activities of membrane-bound enzymes are affected by their lipid environment (*44*).

In addition, we did not find any specific regulatory elements involved in ATP-mediated activation either in the granular soluble contents or in parotid cytosol. Indeed, both the granular soluble contents and parotid cytosol facilitated ATP-mediated activation of the granular membrane-associated PLA_2, but this was not strictly specific to these cellular fractions, as BSA mimicked this effect. Based on these observations, ATP may interact with the granular PLA_2 directly, as is the case with other well-documented Ca^{2+}-independent PLA_2s (*27, 36, 37, 40*).

The physiological significance of ATP-mediated activation is a matter of controversy even for well-documented Ca^{2+}-independent PLA_2s. Hazen and Gross reported that the 40-kDa catalytic subunit of myocardial Ca^{2+}-independent PLA_2 was associated with phosphofructokinase, and suggested its coordinate regulation by ATP with glycolysis (*37*). In contrast, Dennis and colleagues questioned the *in vivo* regulatory role of ATP for P388D_1 PLA_2, because the effect of ATP was dependent on the presence of Triton X-100 and was manifested by several other nucleotides (*27, 40*). In addition, a cloned Ca^{2+}-independent PLA_2 in Chinese hamster ovary cells was hardly activated by ATP, although this enzyme closely resembled P388D_1 PLA_2 in other biological and genetic properties (*27*). Dennis and colleagues suggested that ATP stabilizes and protects the enzyme during *in vitro* assay.

At present, it is unclear whether ATP has a regulatory effect on parotid granular PLA_2 *in vivo*. However, we clearly demonstrated that the nucleotide-mediated activation was restricted to purine nucleotide triphosphates, especially ATP. Unlike myocardial and P388D_1 PLA_2, a nonhydrolyzable analogue of ATP, ATPγS, was much less effective, and ADP and AMP had no effect. In addition, the granular PLA_2 was not denaturated during incubation even in the absence of ATP, since the apparent activity was linear with time for at least 60 min at 37°C (*15*). These findings suggest that the effect of ATP is not due solely to stabilization of the enzyme *in vitro*.

In the salivary gland, ATP is important as an energy donor for saliva secretion. During secretion, the apparent concentration of ATP in the gland was reported to be unchanged (around 1 mM) or slightly decreased (*45, 46*), although metabolic energy supplied by hydrolysis of ATP was increased by 7–8-fold. These observations suggest that ATP is also produced actively to compensate for its rapid consumption. This ATP may have a regulatory effect on parotid granular PLA_2 *in vivo*.

The present results demonstrated the presence of an ATP-stimulatable, Ca^{2+}-independent PLA_2 in rat parotid secretory granular membranes, which seemed to be different from other well-documented Ca^{2+}-independent PLA_2 in its unsusceptibility to various inhibitors. Further investigations including purification and molecular cloning of the granular enzyme are necessary to clarify the mechanism of ATP-mediated activation and its physiological functions such as secretion.

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