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Role of Ca^{2+}-Independent Phospholipase A2 in Exocytosis of Amylase from Parotid Acinar Cells[1]

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We evaluated the role of cytosolic phospholipase A2 (PLA2) in the exocytosis of amylase from parotid acinar cells. The exocytosis stimulated by isoproterenol was dose-dependently inhibited by bromoenol lactone (BEL), a potent suicide inhibitor of Ca^{2+}-independent cytosolic PLA2. The IC_{50} value of BEL was approximately 7 μM. AACOCF3, a selective inhibitor of Ca^{2+}-dependent cytosolic PLA2, did not inhibit the exocytosis at least up to 30 μM. BEL also inhibited amylase release evoked by forskolin and membrane-permeable cAMP, but it did not inhibit cAMP-dependent protein kinase activity. PLA2 activity in parotid acinar cells was found to be predominantly Ca^{2+}-independent, and was strongly inhibited by BEL, whose IC_{50} value was approximately 2 μM when it was applied to intact acini. Although isoproterenol scarcely enhanced [^{3}H]arachidonic acid release from intact acinar cells, BEL dose-dependently decreased the basal arachidonic acid release to approximately one half of the control value. These results suggest that the cytosolic Ca^{2+}-independent PLA2 activity plays a role in the membrane fusion process of exocytosis in parotid acinar cells.

Key words: Ca^{2+}-independent phospholipase A2, cytosolic phospholipase A2, exocytosis, parotid acinar cell, phospholipase A2 inhibitor.

Exocytosis is a process in which the membrane of secretory granules fuses with the plasma membrane. According to the prevailing theory (SNARE hypothesis), specific proteins on the secretory granule membrane and on the plasma membrane form a complex with cytosolic proteins that have weak ATPase activity (*1, 2*). The specific protein-protein interaction is recognized to dock secretory granules to their proper destination. However, little is known concerning the component that is responsible for the final membrane fusion.

Phospholipase A2 (PLA2) has long been postulated to be involved in the membrane fusion process that leads to various types of intracellular vesicle traffic, including exocytosis, by perturbing phospholipid bilayers or by providing fusogenic substances such as arachidonic acid or its metabolites. Namely, eicosanoids and stimulators of PLA2 activity, such as mellitin and mastoparan, induced exocytosis in various cells (*3-6*), including parotid acinar cells (*7*). Inhibitors of PLA2 block exocytosis (*8-10*). In addition, phospholipase A2 promotes fusion between secretory granules and plasma membranes in cell-free reconstitution systems prepared from various secretory cells (*11-13*), including parotid acinar cells (*14*). In those experiments, however, it was not established what type of PLA2 is actually involved in the exocytotic membrane fusion under physiological conditions.

Most cells contain at least two types of intracellular PLA2: Ca^{2+}-dependent and Ca^{2+}-independent. The Ca^{2+}-dependent cytosolic PLA2 has a molecular mass of 85 kDa, and its cDNA has been cloned from human U937 cells (*15*). The enzyme is activated and translocated from the cytosol to the membrane fraction in response to an increase in intracellular Ca^{2+} concentration (*16, 17*). It is also activated by phosphorylation with MAP kinase (*18*). On the other hand, Ca^{2+}-independent PLA2 was purified from canine myocardium (*19, 20*) and P388D1 macrophage-like cells (*21*). The enzymes from both sources form high-molecular-mass complexes (~400 kDa) with other proteins, such as phosphofructokinase (*21, 22*), and share similar enzymatic properties. However, the molecular masses of their catalytic subunits are completely different; the PLA2 of myocardium is 40 kDa and that of P388D1 cells is 80 kDa. In addition, low concentrations of nonionic detergents completely inhibit myocardial PLA2, but markedly activate the enzyme of P388D1 cells.

Although these Ca^{2+}-dependent and Ca^{2+}-independent cytosolic PLA2 must play important roles in the regulation of diverse cellular functions, these roles have not been well distinguished. To gain insight into these issues, we can now utilize highly selective inhibitors for these PLA2s: arachidonyl trifluoromethyl ketone (AACOCF3) (*23-25*) and a bromoenol lactone (BEL) (*26*) for Ca^{2+}-dependent and Ca^{2+}-independent PLA2, respectively. Thus, by using these PLA2 inhibitors we have investigated the role of

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Abbreviations: PLA2, phospholipase A2; cps-cAMP, 8-chlorophenylthio-cAMP; AACOCF3, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; MEM, minimum essential medium.

intracellular PLA2 activities in the exocytosis of amylase from parotid acinar cells.

EXPERIMENTAL PROCEDURES

Materials—BEL and AACOCF3 were purchased from Biomol (Plymouth Meeting, PA., USA) and Signal Transduction (San Diego, CA), respectively. L-α-1-Palmitoyl-2-arachidonyl[arachidonyl-1-^{14}C]phosphatidylcholine and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid were from Dupont NEN-Daiichi (Tokyo) and Amersham (Little Chalfont, England), respectively. PKA assay kit (SpinZyme) was from Pierce (Rockford, IL). Pefabloc SC was from Merck (Darmstadt, Germany). All other chemicals utilized were of the highest grade commercially available.

Assay of Amylase Release—Rat parotid acini (small acini) were prepared as described previously (*27*). Suspensions of parotid acini were preincubated with various concentrations of BEL or its vehicle (DMSO) at 37°C for 15 min in minimum essential medium buffered with 20 mM Na-Hepes (pH 7.4) containing 0.1% BSA (MEM-H). Acini (1 ml) were then pipetted into 1.5-ml microcentrifuge tubes, each containing one of various secretagogues, and incubated at 37°C for 15 min. After incubation each tube was mixed and centrifuged at 10,000 rpm for 1 min, and the supernatant was used for the amylase assay. For measurement of total amylase activity, acini were incubated with 0.2% Triton X-100 as above. Amylase activity was measured by the method of Bernfeld (*28*), and the released amylase activity was expressed as a percentage of the total activity.

Assay of PKA Activity—Parotid acini were incubated with BEL or its vehicle (DMSO) for 15 min at 37°C. Acini were washed twice with MEM-H and then lysed with 50 mM Na-Hepes (pH 7.4), 1% Triton X-100, and 0.2 mM Pefabloc SC at 0°C for 10 min. The lysate was centrifuged at 15,000 rpm for 5 min, and the supernatant was used as an enzyme source. PKA activity was measured by use of a PKA assay kit (SpinZyme), according to the instructions supplied with the kit.

Assay of PLA2 Activity—Parotid acini were incubated for 15 min at 37°C in the presence or absence of BEL. Acini were washed twice with MEM-H and homogenized in a Teflon-glass homogenizer with 20 mM Na-Hepes buffer (pH 7.4) containing 0.2 mM Pefabloc SC. The homogenate was centrifuged at 18,000 × g for 20 min at 4°C.

The secretory granule fraction was prepared as described previously (*29*). Briefly, parotid glands removed from a rat starved overnight were homogenized in a Teflon-glass homogenizer with 0.25 M sucrose, 10 mM Na-Hepes (pH 7.4), 1 mM EGTA, and 0.2 mM Pefabloc SC at room temperature. The homogenate was centrifuged at 150 × g for 5 min, and the supernatant was taken and centrifuged at 1,130 × g for 10 min at room temperature to obtain the secretory granule fraction (pellet), which was then washed once with the sucrose medium. The supernatant obtained was centrifuged at 123,000 × g for 60 min at 4°C.

PLA2 activity in the cytosolic fraction and secretory granule fraction was measured with L-α-1-palmitoyl-2-arachidonyl[arachidonyl-1-^{14}C]phosphatidylcholine as a substrate. The phosphatidylcholine was dried under N_2 and then sonicated with water. The reaction mixture (0.2 ml) was composed of 100 mM Na-Hepes (pH 7.4), 2 μM (0.023 μCi) [^{14}C]phosphatidylcholine, various concentrations of Ca^{2+}, and the enzyme sample (±0.05% Triton X-100). The mixture was incubated for 30 min at 37°C, and the reaction was terminated by the addition of 1 ml of Dole reagent (isopropanol/heptane/1 N H_2SO_4, 78 : 20 : 2). Heptane (0.6 ml) and water (0.4 ml) were then added to the mixture, which was mixed vigorously and centrifuged at 3,000 rpm for 5 min. The upper phase (0.6 ml) was removed and mixed with 0.6 ml of heptane and 100 mg silica gel and centrifuged at 3,000 rpm for 5 min. The radioactivity of heptane (0.8 ml) was measured with a scintillation counter.

Assay of Arachidonic Acid Release—Parotid acini were prelabeled with [^{3}H]arachidonic acid at 37°C for 60 min in MEM-H containing 0.1% BSA and [^{3}H]arachidonic acid (2.5 μCi/ml). The acini were washed twice with MEM-H containing 1% BSA and then incubated with various concentrations of BEL for 15 min in the same medium. [^{3}H]-Arachidonic acid release from these acini was measured for 15 min in the presence or absence of isoproterenol. After incubation each tube was centrifuged at 15,000 rpm for 1 min, and the radioactivity of the supernatant was determined with a liquid scintillation counter. For measurement of total [^{3}H]arachidonic acid incorporated, acini were lysed with 1% Triton X-100 and the radioactivity of the lysate was measured as above.

RESULTS

Effects of PLA2 Inhibitors on Amylase Release—Amylase release from parotid acinar cells is a typical model of Ca^{2+}-independent and cyclic AMP-mediated exocytosis (*30*). To evaluate the role of cytosolic PLA2 in amylase exocytosis, we preincubated parotid acini with various concentrations of BEL or AACOCF3 and then stimulated amylase release by adding isoproterenol. As shown in Fig. 1, BEL had no effect on the basal amylase release, but dose-dependently inhibited amylase release evoked by isoproterenol. The IC_{50} value of BEL for isoproterenol-

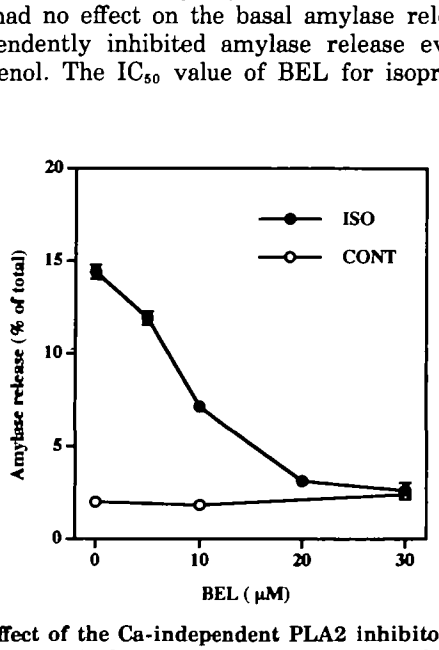

Fig. 1. **Effect of the Ca-independent PLA2 inhibitor BEL on amylase exocytosis from rat parotid acini.** Parotid acini were preincubated with various concentrations of BEL for 15 min at 37°C and further incubated for 15 min after addition of 1 μM isoproterenol or its vehicle. Data shown are means ± SD (n = 3) of a representative experiment from 3 independent experiments.

induced amylase release was approximately 7 μM. On the other hand, as shown in Fig. 2, AACOCF3 did not inhibit amylase release at least up to 30 μM. However, it markedly increased basal release, especially above 30 μM.

To examine whether or not BEL inhibited amylase release through antagonistic effects on β-adrenergic receptors or adenylyl cyclase, we investigated the effect of BEL on amylase release evoked by forskolin, a direct activator of adenylyl cyclase, and by CPS-cAMP, a membrane-permeable cAMP analog. As shown in Fig. 3, BEL clearly inhibited amylase release evoked by both stimuli.

Then we examined the effect of BEL on PKA activity. Parotid acini were incubated with 20 μM BEL or its vehicle DMSO at 37°C for 15 min and washed twice with incubation medium without BEL. PKA activity of the acinar lysate was measured with a nonradioactivity-based assay kit. As shown in Fig. 4, BEL had no inhibitory effect on PKA activity. Similarly no inhibitory effect was detectable even when the cell lysate was directly preincubated with 20 μM BEL at 37°C for 15 min and the PKA activity of the extract was measured in the presence of 8 μM BEL (data not shown). These results suggest that BEL inhibits amylase exocytosis at a site distal to the protein phosphorylation evoked by PKA.

Effect of BEL on PLA2 Activity—Since BEL is a potent suicide inhibitor of Ca^{2+}-independent cytosolic PLA2 (*26*),

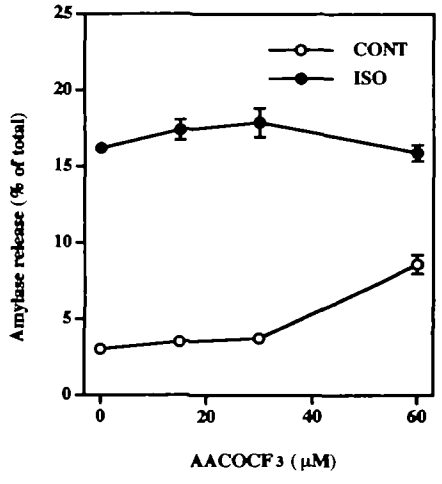

Fig. 2. **Effect of Ca-dependent PLA2 inhibitor AACOCF3 on amylase exocytosis from parotid acini.** Parotid acini were preincubated with various concentrations of AACOCF3 for 5 min at 37°C and further incubated for 15 min after addition of 1 μM isoproterenol or its vehicle. Data shown are means ± SD (n = 3) of a representative experiment from 4 independent experiments.

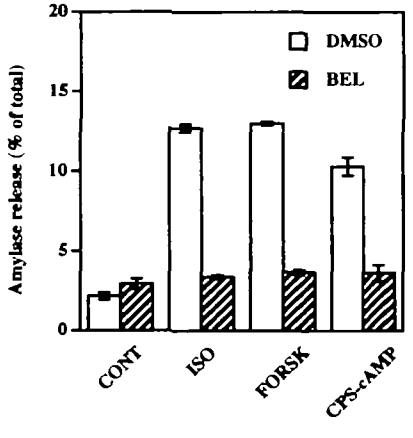

Fig. 3. **Effect of BEL on amylase release from parotid acini stimulated separately by isoproterenol, forskolin, and cps-cAMP.** Parotid acini were preincubated with 20 μM BEL for 15 min at 37°C and further incubated for 15 min after addition of 1 μM isoproterenol, 10 μM forskolin, or 1 mM cps-cAMP. Data shown are means ± SD (n = 3) of a representative experiment from 2 independent experiments.

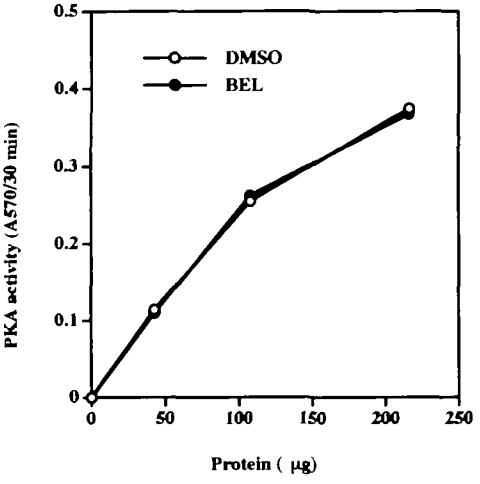

Fig. 4. **Lack of effect of BEL on cAMP-dependent protein kinase (PKA) activity in parotid acini.** Parotid acini were incubated with 20 μM BEL or its vehicle for 15 min at 37°C. After incubation, acini were washed twice and cell lysates were prepared as described in "EXPERIMENTAL PROCEDURES." PKA activity was measured by use of a PKA assay kit (SpinZyme) according to its protocol. Data shown are means of duplicate determinations in a representative experiment from 3 independent experiments.

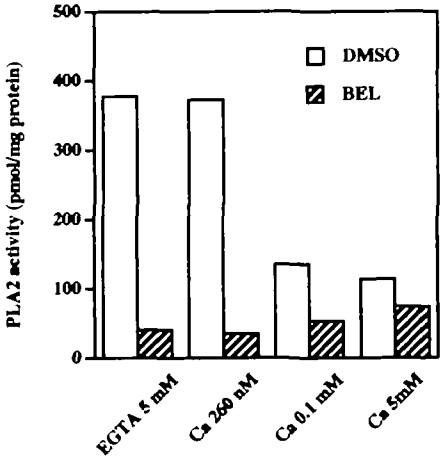

Fig. 5. **Effects of Ca^{2+} concentration and BEL treatment on PLA2 activity in parotid acinar cells.** Parotid acini were incubated with or without 20 μM BEL for 15 min at 37°C. After incubation, the acini were washed twice and cell extracts were prepared as described in "EXPERIMENTAL PROCEDURES." PLA2 activity was measured at 37°C for 30 min with [^{14}C]phosphatidylcholine as a substrate in the presence of various Ca concentrations. Data shown are means of duplicate determinations in a representative experiment from 4 independent experiments.

we next investigated the correlation between amylase release and PLA2 activity in parotid acini. For that purpose we first examined the Ca^{2+} requirement for PLA2 activity in parotid acini. As shown in Fig. 5, the PLA2 activity was maximum in the absence of Ca^{2+} (5 mM EGTA). PLA2 activity at 260 nM Ca^{2+} (5 mM EGTA + 4 mM $CaCl_2$, pH 7.4) was as strong as that in the absence of Ca^{2+}, but it was markedly reduced in the presence of higher than submillimolar concentrations of Ca^{2+}. Then we examined the effect of BEL on PLA2 activity at various concentrations of Ca^{2+}. BEL (20 μM) strongly but not completely inhibited PLA2 activity. The remaining PLA2 activity was slightly increased in the presence of higher Ca^{2+} concentrations.

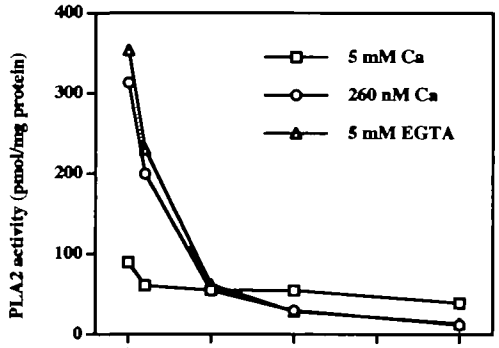

Fig. 6. **Effect of BEL on PLA2 activity in parotid acini.** Parotid acini were incubated with various concentrations of BEL for 15 min at 37°C. After incubation, acini were washed twice and cell extracts were prepared as described in "EXPERIMENTAL PROCEDURES." PLA2 activity was measured in the presence of 5 mM EGTA, 260 nM Ca^{2+}, and 5 mM Ca^{2+}. Data shown are means of duplicate determinations in a representative experiment from 3 independent experiments.

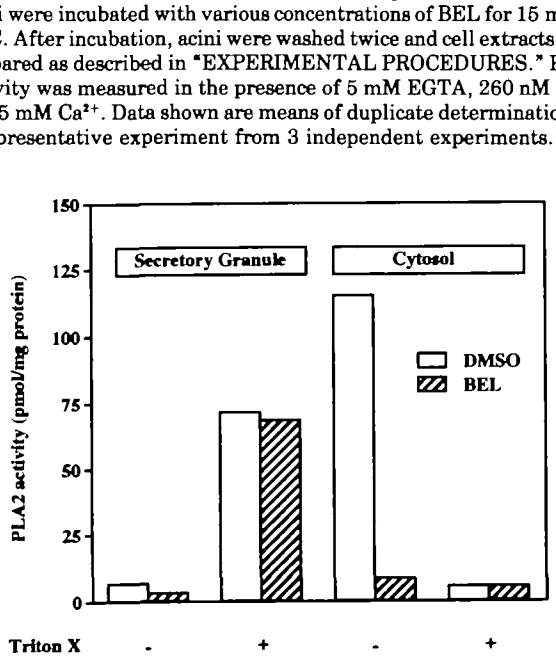

Fig. 7. **Comparison of Ca^{2+}-independent PLA2 activities in the secretory granule fraction and the cytosolic fraction of parotid acinar cells.** Parotid acini were incubated with or without 20 μM BEL for 15 min at 37°C. After incubation, the acini were washed twice and the secretory granule fraction and the cytosolic fraction were prepared as described in "EXPERIMENTAL PROCEDURES." Ca^{2+}-independent PLA2 activity was measured in the presence or absence of 0.05% Triton X-100. Data shown are means of duplicate determinations in a representative experiment from 4 independent experiments.

These results indicate that PLA2 activity in parotid acini was predominantly Ca^{2+}-independent.

BEL dose-dependently inhibited Ca^{2+}-independent PLA2 activity (Fig. 6). For these experiments, parotid acini were incubated with various concentrations of BEL at 37°C for 15 min, washed twice, and then homogenized. PLA2 activity was measured in the presence of 5 mM EGTA, 260 nM Ca^{2+}, or 5 mM Ca^{2+}. In the presence of 5 mM EGTA or 260 nM Ca^{2+}, BEL strongly inhibited PLA2 activity; the IC_{50} value was approximately 2 μM. On the other hand, when PLA2 activity was measured at 5 mM Ca^{2+}, 1 μM BEL clearly decreased the activity, but higher concentrations of BEL scarcely enhanced the inhibition. This suggests that the initial inhibition by 1 μM BEL is due to the inhibition of residual Ca^{2+}-independent PLA2 activity. In other words, Ca^{2+}-dependent PLA2 activity is highly resistant to BEL.

The PLA2 activity in the cytosolic fraction was almost completely inhibited by 0.05% Triton X-100 (Fig. 7). In contrast, as reported previously (*31*), Ca^{2+}-independent PLA2 activity in secretory granule membranes was greatly

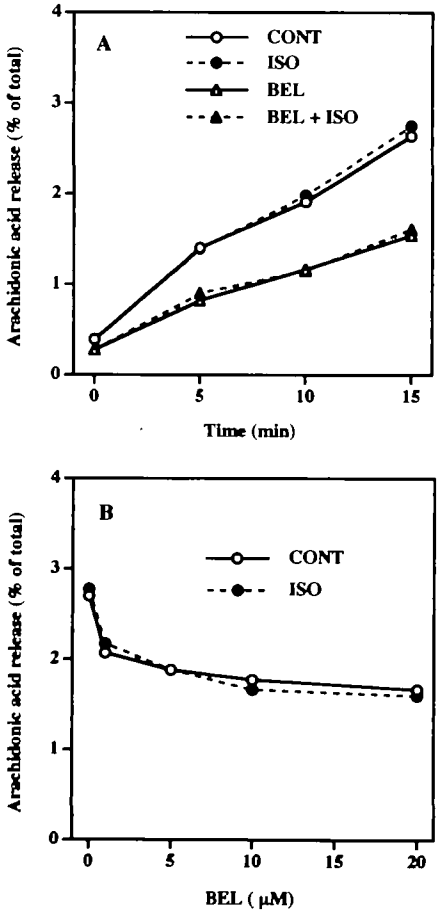

Fig. 8. **[^{3}H]Arachidonic acid release from parotid acinar cells.** Parotid acini were prelabeled with [^{3}H]arachidonic acid for 60 min, preincubated with 0-20 μM BEL for 15 min, and then incubated for 0-15 min in the presence or absence of 1 μM isoproterenol. A: Time course of [^{3}H]arachidonic acid release from the acini preincubated with or without 20 μM BEL. B: Dose response curves of BEL. Acini were incubated for 15 min in the presence or absence of 1 μM isoproterenol. Data shown are means of duplicate determinations in a representative experiment from 3 independent experiments.

activated in the presence of Triton X-100. Furthermore, PLA2 activity of the secretory granule was quite insensitive to BEL.

Effects of BEL on Arachidonic Acid Release—We finally evaluated the effects of BEL and isoproterenol on arachidonic acid release from intact parotid acini. As shown in Fig. 8, A and B, 1 μM isoproterenol scarcely increased arachidonic acid release from the acini that were prelabeled with [^{3}H]arachidonic acid for 60 min, indicating that activation of PLA2 is unlikely to be involved in amylase exocytosis evoked by isoproterenol. However, BEL dose-dependently decreased the basal arachidonic acid release to almost a half of the control value.

DISCUSSION

PLA2 Activity in Parotid Acinar Cells—The present study clearly shows that cytosolic PLA2 activity in parotid acinar cells is predominantly Ca^{2+}-independent. This enzyme does not require Ca^{2+} for its catalytic activity and the maximal activity is given in the absence of Ca^{2+} (5 mM EGTA). The maximum enzyme activity is maintained within the range of physiological Ca^{2+} concentrations, but it is markedly inhibited by a high concentration of Ca^{2+} ($\geq$0.1 mM). It was recently found that calmodulin is involved in the mechanism of inhibition by Ca^{2+} (*32*). Furthermore, BEL almost completely inhibited PLA2 activity under a low Ca^{2+} condition. These characteristics closely resemble those of Ca^{2+}-independent PLA2 activities found in canine myocardium (*19*) and rat hypocampus (*33*).

Mizuno *et al.* (*31*) reported that secretory granule membranes of rat parotid glands contain Ca^{2+}-independent PLA2 activity. In this study we confirmed the existence of the enzyme there. This enzyme is really Ca^{2+}-independent, but is not markedly inhibited by higher Ca^{2+} concentrations (data not shown). As reported, the activity was only detectable in the presence of a low concentration of Triton X-100 (0.05%), although the same concentration of Triton X-100 almost completely inhibited the activity in the soluble fraction. Furthermore, BEL hardly inhibited the activity in the secretory granules. Thus, the two Ca^{2+}-independent PLA2 activities in the cytosol and the secretory granule membrane seem to be very different.

Effect of BEL on Amylase Exocytosis—To our knowledge this is the first report that BEL inhibits exocytosis. Amylase exocytosis from rat parotid acini is mainly stimulated by the cAMP-mediated signaling system (*34, 35*). Although a role for Ca^{2+} in the cAMP-mediated exocytosis is not completely ruled out, it has been recognized that an increase in intracellular Ca^{2+} is not necessary for the exocytosis (*35-38*). Therefore, it is not surprising that BEL but not AACOCF3 inhibited amylase release stimulated by isoproterenol. Although AACOCF3 also inhibits purified Ca^{2+}-independent PLA2 activity in a cell-free assay system (*39*); the IC_{50} value of AACOCF3 is 15 μM, which is 250-fold higher than that of BEL. Thus, 30 μM AACOCF3 used in this study would be insufficient for inhibition of Ca^{2+}-independent PLA2 activity in intact cells. BEL also inhibited amylase release induced by forskolin or membrane-permeable cAMP analogs, indicating that BEL is unlikely to act as an antagonist of β-receptors or adenylyl cyclase. In addition, since BEL did not inhibit PKA activity, BEL inhibits amylase exocytosis at a site distal to the protein phosphorylation evoked by PKA.

Previously BEL was found to inhibit endosome fusion in a cell-free reconstitution system (*40*). Similarly, a wide variety of PLA2 inhibitors blocked intra-Golgi transport *in vitro* (*41*). More recently, plasma membrane that had been pretreated with phospholipase A2 was shown to undergo increased Ca^{2+}-independent fusion with secretory granules of parotid acini (*14*). These results and our present findings collectively suggest that Ca^{2+}-independent PLA2 promotes the fusion between the plasma membrane and the membrane of secretory granules by perturbing phospholipid bilayers or by providing fusogenic substances such as arachidonic acid or its metabolites.

However, it is noteworthy that the dose-response curves of BEL for amylase release (Fig. 1) and PLA2 activity (Fig. 6) are not completely correlated. BEL inhibited PLA2 activity more strongly than amylase release. At 5 μM BEL, PLA2 activity was inhibited by 80%, but amylase release was inhibited only by 20%. This suggests that only a small portion of PLA2 activity is sufficient to maintain most of the amylase exocytosis. On the other hand, the effect of BEL on [^{3}H]arachidonic acid release was less prominent; 20 μM BEL inhibited the basal arachidonic acid release by less than 50% (Fig. 8), although the characteristics of the remaining arachidonic acid release were not established. We consider that the exocytotic process in parotid acini is fairly resistant to exogenous inhibitors. In other words, the exocytosis must be very sensitive to endogenous stimuli.

At present, the regulatory mechanism of Ca^{2+}-independent cytosolic PLA2 activity is mostly unknown. The purified enzyme was reported to be activated by ATP without phosphorylation (*42, 43*). However, we could not detect the activation when 1 mM ATP was added to the reaction mixture (data not shown), probably because the crude enzyme in the homogenate is already activated. Moreover, isoproterenol did not increase [^{3}H]arachidonic acid release (Fig. 8), suggesting that activation of PLA2 activity is unlikely to be involved in cAMP-mediated exocytosis. Ca^{2+}-independent PLA2 activities were found to be associated with high-molecular-weight complexes (*21, 22*), including those containing phosphofructokinase. Thus we are now interested in the relationship between Ca^{2+}-independent PLA2 and the docking-fusion complex (SNARE complex) in parotid acinar cells. When the v-SNARE on the secretory granule membrane and the t-SNARE on the plasma membrane form a complex with cytosolic proteins, Ca^{2+}-independent PLA2 may well be associated with the complex and thus promote the fusion of the two membranes. Further study is necessary to test this hypothesis.

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