Ca²⁺-independent fusion of secretory granules with phospholipase A₂-treated plasma membranes *in vitro*

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The fusion of secretory granules with plasma membranes prepared from rat parotid gland was studied *in vitro* to clarify the mechanism of exocytosis. Fusion of the granules with plasma membranes was measured by a fluorescence-dequenching assay with octadecyl rhodamine B, and release of amylase was also measured to confirm the fusion as a final step of the secretory process. Plasma membranes that had been pretreated with porcine phospholipase A₂ (PLA₂) in the presence of 20 μ M Ca²⁺ fused with the granules within 30 s, and induced amylase release by reacting with the membranes of granules, whereas without this pretreatment they had no significant effect. The fusion process accompanied by amylase release was induced in the presence of 10 mM EGTA, and therefore was apparently Ca²⁺-independent. On the other hand, the presence of EGTA or 100 μ M quinacrine, an inhibitor of PLA₂, during treatment of plasma membranes with PLA₂ inhibited their fusogenic activity,

suggesting the importance of activation of PLA₂. Arachidonic acid and linoleic acid were released from the plasma membranes during the PLA₂ treatment. The presence of albumin, an adsorbent of fatty acids, during the treatment also inhibited the activity. Pretreatment of the membranes with arachidonic acid or linoleic acid did not have any effect, but the presence of exogenously added arachidonic acid during PLA₂ treatment enhanced the membrane-fusion-inducing effect of PLA₂. Pretreatment of the membranes with lysophosphatidylcholine induced fusogenic activity. These findings suggest that the conformational change in the plasma-membrane phospholipids induced by PLA₂ and the presence of arachidonic acid or linoleic acid produced by PLA₂ are important in the process of fusion of secretory granules with the plasma membranes of rat parotid acinar cells and that the fusion process itself is independent of Ca²⁺.

INTRODUCTION

Secretion by endo- and exo-crine cells is known to occur by exocytosis, but the precise mechanism of fusion of secretory granules with the plasma membrane is not known. Close contact of two lipid bilayers does not induce their fusion; in fact they repel each other [1]. This suggests that some intracellular factor(s) is required for the fusion process to take place. Synexin, a Ca²⁺/phospholipid-binding protein of the annexin family, has been reported to promote Ca²⁺-dependent fusion of chromaffin granules [2–4], lamellar bodies of lung surfactant [5] and an artificial bilayer and a natural membrane [6]. Activation of protein kinase C stimulated binding of synaptic vesicles to the presynaptic membrane [7] and exocytosis from adrenal chromaffin cells [8–10]. Exo 1 and Exo 2, two cytosolic proteins, stimulated exocytosis in permeabilized chromaffin cells [11]. Interaction between protein kinase C and Exo 1 has also been reported [12,13]. Regulation of exocytosis in adrenal chromaffin cells by GTP-binding protein(s) has been suggested [14–19]. Syntaxin, a 35 kDa protein expressed in the nervous system, was suggested to function in the fusion of synaptic vesicles at presynaptic active zones [20]. Over the past few years, synaptic-vesicle proteins such as synaptophysin, synaptotagmin and synaptobrevin have been intensely studied (for reviews see refs [21,22]). More recently, the Rab 3A-Rab GDI-Rabphilin 3 system (for a review see ref. [23]) and NSF-SNAP-SNARE system [24] have also been suggested to be involved in membrane fusion in the synapse. Thus a great deal of information about the

proteins that regulate vesicle docking at the plasma membrane has been gathered.

Stimulation of bovine adrenal chromaffin cells with acetylcholine increased the intracellular Ca²⁺ concentration and resulted in release of large amounts of catecholamines together with free arachidonic acid [25,26]. Exogenous arachidonic acid increased catecholamine secretion from bovine adrenal chromaffin cells [27,28]. On the other hand, inhibitors of phospholipase A₂ (PLA₂) were reported to inhibit the release of arachidonic acid and catecholamine secretin [29]. This circumstantial evidence strongly suggests a pivotal role for PLA₂ in the secretory mechanism of chromaffin cells. The stimulatory effects of melittin and mastoparan, found in honey bee venom and wasp venom respectively, on secretion in many cell types [30–35] support this role for PLA₂, because these compounds have stimulatory actions on PLA₂ [36–38] as well as on GTP-binding protein [37–40] and phospholipase C [41,42]. Furthermore, Izumi et al. [43] clearly and simply showed the involvement of PLA₂ in catecholamine secretion using a cell-free model system.

In 1990, Karli et al. [44] first reported direct evidence of fusion of chromaffin granules with the plasma membrane by an elegant method of fluorescence dequenching of the lipid marker, octadecyl rhodamine B (R18), as developed by Hoekstra et al. [45]. This method was subsequently confirmed by several other studies [46–48] to be valid. The method is based on the principle of dequenching the fluorescence signal of a marker that has been incorporated into the membrane phospholipids of chromaffin granules.

Abbreviations used: PLA₂, phospholipase A₂; R18, octadecyl rhodamine B; ETYA, eicosatetrayenoic acid; AA861, 2-(12-hydroxydodeca-5,10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone.

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In the present work, we studied exocytosis of secretory granules of rat parotid acinar cells in a cell-free system, using isolated secretory granules and plasma membranes from rat parotid glands. The fusion of the two was measured by the R18 fluorescence-dequenching assay and secretion of amylase. Our results demonstrated Ca^{2+} -independent fusion induced by PLA_2 -treated membranes.

MATERIALS AND METHODS

Materials

R18 was purchased from Molecular Probes (Eugene, OR, U.S.A.). Porcine pancreatic PLA_2 was from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Quinacrine dihydrochloride, BSA, arachidonic acid and indomethacin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Eicosatetrayenoic acid (ETYA), EGTA, Pipes, Tris and 2-(12-hydroxydodeca-5,10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone (AA861) were from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of analytical grade.

Preparation of secretory granules and plasma membranes of rat parotid acinar cells

Secretory granules and plasma membranes were prepared by the method of Schramm and Danon [49]. Male Wistar rats (250–350 g) were lightly anaesthetized with ether, stunned by a blow on the head, and bled via the carotid arteries. Parotid glands with surrounding tissues were transferred to ice-cold 0.25 M sucrose solution. Connective tissue, fat and lymph glands were removed. Then the glands were weighed and cut into pieces, which were homogenized in 0.25 M sucrose (10 ml/g wet wt.) with a glass/Teflon homogenizer, rotating at 400–500 rev./min by four up and down strokes. The homogenate was filtered through a nylon net (2 mm mesh), and the filtrate was centrifuged at 550 *g* for 10 min to remove unbroken cells as precipitate (P1). P1 was resuspended in 10 ml of 0.25 M sucrose and re-centrifuged at 550 *g*. The two supernatants were combined and centrifuged at 1450 *g* for 10 min to separate a plasma-membrane-enriched supernatant (S2) and a precipitate of secretory granules (P2). The supernatant (S2) was centrifuged at 16000 *g* for 15 min to remove various small intracellular organelles. A crude plasma-membrane fraction was finally obtained as a pellet by ultracentrifugation at 75000 *g* for 40 min. Contaminating sucrose was removed by resuspending the fraction in Pipes buffer (pH 7.2), consisting of 20 mM Pipes, 139 mM potassium glutamate, 5 mM glucose and 5 mM EGTA, and further ultracentrifugation. The precipitate was resuspended in a small volume (< 2 ml) of Pipes buffer containing 20 μM free Ca^{2+} ion (Ca^{2+} /Pipes buffer: the free Ca^{2+} concentration was adjusted with Ca^{2+} /EGTA) and the suspension kept frozen at -70°C .

The crude secretory granule fraction 'P2' was washed once with 0.25 M sucrose and resuspended in 600 μl of 0.25 M sucrose. The turbidity of an aliquot of the suspension was measured and the suspension was mixed with sufficient 0.25 M sucrose to give a constant protein concentration of about 550 μg of protein/ml. All procedures described above were carried out at 4°C . Freshly prepared secretory granules were used in all experiments.

Treatment of plasma membrane with PLA_2

The frozen plasma membranes were thawed, diluted with Ca^{2+} /Pipes buffer to make a suspension of 100 μg of protein/ml, and then incubated with or without PLA_2 at various concentrations

(0.055–7 units/ml) for 30 min at 37°C . Then an equal volume of ice-cold Pipes buffer was added and the mixtures were centrifuged at 100000 *g* for 30 min. PLA_2 -treated and untreated membranes were resuspended in reaction mixture, consisting of (in mM) KCl 111.1, MgCl_2 3.33, sucrose 22.2, Tris/HCl buffer (pH 7.4) 11.1 and EGTA 1.11, at a concentration of 400 μg of protein/ml. The suspensions were kept frozen at -70°C until use.

Loading of secretory granules with fluorescence dye

The suspension of secretory granules prepared as described above was diluted with 9 vol. of reaction mixture and incubated with 20 μM R18 for 6 min at 37°C in the dark. R18-loaded secretory granules were precipitated by centrifugation at 1450 *g* for 10 min, resuspended in their original volume of 0.25 M sucrose solution and kept on ice in the dark until use.

Measurement of amylase release from secretory granules

The reaction was started by addition of 15 μl of secretory-granule suspension prepared as described above to 135 μl of reaction mixture containing various concentrations of plasma membranes (2.4–19.2 μg of protein). The incubation was carried out in Ca^{2+} -free conditions in the presence of 1 mM EGTA. After incubation for 4 min, the reaction was stopped by centrifugation at 1450 *g* for 10 min. The activity of amylase released into the supernatant was measured by the method of Bernfeld [50] with amylose as substrate. Activity was expressed as mg of maltose liberated into the medium/5 min at 20°C and also as a percentage of the total activity.

Measurement of fusion of secretory granules with plasma membranes

A 30 μl volume of R18-loaded secretory granule suspension was mixed with 120 μl of 0.25 M sucrose and 1302 μl of reaction mixture in a cuvette maintained at 37°C . The reaction was started by addition of 48 μl (19.2 μg of protein) of plasma-membrane suspension. R18 fluorescence was monitored using excitation and emission wavelengths of 560 nm and 590 nm respectively in a Hitachi F-2000 fluorescence spectrophotometer. Increase in fluorescence intensity induced by dequenching on fusion of the granules with plasma membranes is expressed in arbitrary units as the increase from the basal level, i.e. the level before addition of plasma membranes. In preliminary experiments, net increase in fluorescence intensity induced by incubation of R18-loaded granules with membranes (treated with 7 units of PLA_2 ; 19.2 μg of protein) at 37°C for 3 min was $5.4 \pm 0.9\%$ ($n = 10$) of the fluorescence obtained at an infinite dilution of the R18 after addition of detergent (0.2% Triton X-100).

Measurement of non-esterified fatty acids liberated from membrane preparation

After incubation of plasma membranes with porcine PLA_2 , non-esterified fatty acids released from plasma membranes into the incubation medium and heptadecanoic acid, which was added to the medium as an internal standard, were extracted with chloroform/methanol (1:2, v/v) and chloroform/aq. 0.25 M KCl + 5 mM EDTA (1:1, v/v). The chloroform layer was taken and evaporated to dryness under vacuum. The non-esterified fatty acids in the dried material were redissolved with methanol and allowed to react with 9-anthryldiazomethane, a fatty acid-labelling fluorescent reagent, to produce the fluorescent ester

derivatives. The esters were assayed after HPLC separation (Intersil ODS-2 column: 4.6 mm × 250 mm; GL Sciences, Tokyo, Japan; Yanaco L-5000 liquid chromatography equipped with Shimadzu RF-535 spectrofluorophotodetector and Hitachi D-2500 chromatointegrator) by fluorescence detection at 412 nm with excitation at 365 nm [51].

Procedures for light and electron microscopy

Secretory granules and plasma membranes were precipitated by centrifugation and fixed overnight at 4 °C with 2.5 % glutaraldehyde/2 % paraformaldehyde containing 0.1 M cacodylate buffer (pH 7.4). The fixed materials were rinsed with 0.1 M cacodylate buffer containing 0.05 % CaCl_2 and 4 % sucrose at 4 °C, and post-fixed for 1 h at 4 °C with 1 % OsO_4 containing 0.1 M cacodylate buffer. They were then dehydrated by passage through a series of increasing concentrations of ethanol and embedded in Epon 812. Sections of 1 μm thickness were prepared and stained with 0.1 % Methylene Blue containing 10 % ethanol and 0.01 % KOH for light microscopy. Ultrathin sections with a pale-gold interference colour were prepared and stained with uranyl acetate and lead citrate; and examined under an electron microscope (Hitachi, H-600).

Statistical analysis

Results were analysed statistically by Student's *t* test and *P* values of less than 0.05 were regarded as significant.

RESULTS

Release of amylase induced by fusion of secretory granules with plasma membranes prepared from rat parotid

Secretory granules released about 27 % of their total amylase on incubation at 37 °C for 30 s. Without pretreatment, plasma membranes had no significant effect on this release. Increasing the incubation time to 3 min did not increase the amount released (Table 1). However, in plasma membranes that had been treated with porcine PLA_2 in the presence of 20 μM Ca^{2+} for 30 min at 37 °C amylase release significantly increased (Table 1). The stimulatory effect was almost maximum after 30 s, further release during the 3 min incubation being very slight, suggesting a rapid effect of the PLA_2 -treated membranes. The results obtained with 9.6 μg of membrane protein are shown in Table 1; similar results

Table 1 Effect of treatment of plasma membranes with PLA_2 on amylase release from secretory granules of rat parotid glands

Secretory granules were incubated without or with plasma membranes or with PLA_2 -treated plasma membranes at 37 °C for 30 s or 3 min under Ca^{2+} -free conditions. Plasma membranes were pretreated with 7 units/ml PLA_2 in the presence of 20 μM Ca^{2+} . The total amylase activity of secretory granules was 16.4 ± 0.8 mg of maltose/mg of protein ($n = 14$). Values for amylase release are means $\pm$ S.E.M. from three experiments. *Significantly different from the value without plasma membranes, $P < 0.01$. Details of experimental conditions are described in the Materials and methods section.

	Amylase released (% of total)	
	30 s	3 min
Without plasma membranes	27.2 ± 3.3	29.9 ± 1.7
With plasma membranes	27.0 ± 2.6	27.5 ± 7.2
With PLA_2 -treated plasma membranes	$69.0 \pm 2.7^*$	$78.9 \pm 0.6^*$

Figure 1 Effect of the amount of PLA_2 -treated plasma membranes on amylase release from secretory granules

Secretory granules were incubated without ($\square$) or with ($\bullet$) various amounts of PLA_2 -treated membrane (2.4–19.2 μg of protein) or with untreated membranes (9.6 μg , $\circ$) at 37 °C for 30 s. Values are means $\pm$ S.E.M. from three experiments. *Significantly different from the value without plasma membrane, $P < 0.01$. For further details, see the Materials and methods section and the legend of Table 1.

Figure 2 Time course of fusion of secretory granules with PLA_2 -treated plasma membranes

Secretory granules preloaded with the fluorescence dye R18 were incubated without ($\circ$) or with plasma membranes treated with 7 ($\bullet$), 1.75 ($\blacktriangle$), 0.175 ($\blacklozenge$) or 0.0553 ($\blacksquare$) units/ml PLA_2 in the presence of 20 μM Ca^{2+} . The amount of plasma membranes was 19.2 μg of protein. Values are means $\pm$ S.E.M. from three experiments. Inset: a typical trace of fluorescence increase induced by PLA_2 -treated membranes. The treated membranes were added to secretory granules at the point indicated by an arrowhead. For further details, see the Materials and methods section.

were obtained with all amounts of membrane protein tested. Amylase release increased with the amount of PLA_2 -treated plasma membranes added (Figure 1).

Next, we examined the fusion of plasma membranes with

Figure 3 Effect of PLA₂ activity during plasma-membrane treatment on fusion of secretory granules with plasma membranes (a) and amylase release from secretory granules (b)

Plasma membranes [19.2 μ g in (a) and 9.6 μ g in (b)] were treated with various activities of PLA₂ in the presence of 20 μ M Ca²⁺. Secretory granules were incubated with the plasma membranes at 37 °C for 3 min. Values are means $\pm$ S.E.M. from three and five experiments in (a) and (b) respectively. For further details, see the Materials and methods section and the legends of Table 1 and Figure 2.

secretory granules preloaded with R18 by a fluorescence-dequenching assay. On fusion with unloaded plasma membranes, intermixing of the membrane lipids causes dilution of the fluorescent marker, resulting in an increase in fluorescence intensity due to dequenching, which can be measured in a fluorescence spectrometer [45]. Untreated membranes, even with the maximum amount tested, had no appreciable effect on the fluorescence signal, but PLA₂-treated membranes increased the signal significantly within 30 s and then more gradually during the 3 min reaction (Figure 2). Thus PLA₂-treated membranes induced parallel increases in amylase release and fusion of granules with membranes.

As mentioned in the Materials and methods section, the incubation mixtures for studying amylase release and fusion contained 1 mM EGTA. Therefore the process of fusion of secretory granules with plasma membranes followed by amylase release did not seem to require Ca²⁺ and similar fusion followed by amylase release was observed in the presence of 20 μ M Ca²⁺ (results not shown). Increasing the activity of PLA₂ used for plasma-membrane treatment resulted in increased stimulatory effects of the membranes on the fluorescence signal (Figures 2 and 3a) and amylase release (Figure 3b). The fluorescence signal and amylase release were not changed at 0.175 units/ml, but were both increased significantly at 1.75 units/ml, indicating parallel changes in the two parameters.

Liberation of fatty acids from plasma membranes by PLA₂ treatment

Measurement of non-esterified fatty acids liberated into the medium during PLA₂ treatment of plasma membranes in the presence of 20 μ M Ca²⁺ revealed that plasma membranes released unsaturated fatty acids, arachidonic acid and linoleic acid, depending on the activity of PLA₂ used (Figure 4). However, the treatment did not liberate oleic acid (Figure 4) or saturated fatty

Figure 4 Liberation of non-esterified fatty acids from plasma membranes during incubation with PLA₂

Plasma membranes (200 μ g of protein) were incubated without or with various activities of PLA₂ for 30 min at 37 °C. Liberated arachidonic acid (○), linoleic acid (●) and oleic acid (△) are expressed as pmol/200 μ g of membrane protein. *Significantly different from the value without PLA₂, $P < 0.05$. Values are means $\pm$ S.E.M. from three experiments. For further details, see the materials and methods section.

acids such as myristic acid, palmitic acid and stearic acid (not shown).

Effects of EGTA, quinacrine and albumin on the treatment of plasma membranes with PLA₂

In all the experiments described above, plasma membranes were treated with PLA₂ in the presence of 20 μ M Ca²⁺. However, plasma membranes treated with PLA₂ in the presence of 10 mM

Figure 5 Effects of PLA₂ treatment of plasma membranes in the presence of EGTA, quinacrine or albumin on fusion and amylase release

Plasma membranes were treated with 7 units/ml PLA₂ in the presence of 10 mM EGTA, 100 μ M quinacrine or 100 μ g/ml albumin in the presence of 20 μ M Ca²⁺. Secretory granules were incubated with the plasma membranes at 37 °C for 3 min. The amounts of plasma membranes used for measurement of the fluorescence signal ($\square$) and amylase release ($\square$) were 19.2 and 9.6 μ g of protein respectively. Values are means $\pm$ S.E.M. from three and four experiments on fluorescence intensity and amylase release respectively. *Significantly different from the corresponding values for membranes treated with PLA₂ alone, $P < 0.01$. For further details, see the Materials and methods section.

EGTA instead of 20 μ M Ca²⁺ lost more than 80% of their stimulatory effect on fusion of granules with membranes and on amylase release (Figure 5). These results indicate that the action of PLA₂ that causes the membranes to be fusogenic is Ca²⁺-dependent, although the process of fusion *per se* is Ca²⁺-independent.

The addition of 100 μ M quinacrine, an inhibitor of PLA₂, during PLA₂ treatment of plasma membranes inhibited the development of fusogenic activity (Figure 5). The presence of albumin (100 μ g/ml) during PLA₂ treatment also inhibited the acquisition of fusogenic activity (Figure 5). As albumin is reported to adsorb non-esterified fatty acids [52], this result indicates that arachidonic acid (or its metabolites) released from membrane phospholipids by the enzymic activity of PLA₂ are closely involved in the development of fusogenic activity of the membranes. Therefore we examined the role of arachidonic acid and its metabolites in PLA₂ treatment of the membranes.

Effects of indomethacin, ETYA and AA861 on treatment of plasma membranes with PLA₂

Plasma membranes were treated with PLA₂ in the presence of 2.8 μ M indomethacin, an inhibitor of cyclo-oxygenase, 10 μ M ETYA, an inhibitor of 5- and 12-lipoxygenases, or 10 μ M AA861, a selective inhibitor of 5-lipoxygenase. None of these inhibitors had any significant effect on the stimulatory effect of PLA₂-treated membrane on amylase release (Table 2). From these results together with those obtained with quinacrine and albumin, we concluded that arachidonic acid itself is important in developing the fusogenic activity of PLA₂-treated plasma membranes.

Effects of arachidonic acid on treatment of plasma membranes with PLA₂

Plasma membranes that had been pretreated with 10 μ M arachidonic acid alone did not increase the fluorescence signal and

Table 2 Effect of PLA₂ treatment of plasma membranes in the presence of indomethacin, ETYA or AA861 on amylase release

Plasma membranes (9.6 μ g of protein) were treated with 1.75 units/ml PLA₂ in the presence of 2.8 μ M indomethacin, 10 μ M ETYA or 10 μ M AA861 in the presence of 20 μ M Ca²⁺. Secretory granules were incubated with the plasma membranes at 37 °C for 3 min. Values ($n = 3$) obtained with these antagonists were not significantly different from the value for treatment with PLA₂ only.

Treatment	Amylase released (% of total)
None	24.5 $\pm$ 0.9
PLA ₂	71.1 $\pm$ 3.3
PLA ₂ + indomethacin	64.5 $\pm$ 5.0
PLA ₂ + ETYA	62.5 $\pm$ 0.8
PLA ₂ + AA861	64.2 $\pm$ 2.1

Figure 6 Effect of arachidonic acid on PLA₂ treatment of plasma membranes

Plasma membranes were treated with 0.055 or 1.75 units/ml PLA₂ in the presence of 20 μ M Ca²⁺ in the absence or presence of 10 μ M arachidonic acid. Secretory granules were incubated with the plasma membranes at 37 °C for 3 min. The amounts of plasma membrane used were 19.2 μ g of protein for measurement of fluorescence signals ($\square$) and 9.6 μ g of protein for assay of amylase release ($\square$). values are means $\pm$ S.E.M. from three and four experiments on fluorescence intensity and amylase release respectively. Significance of difference from the corresponding value for PLA₂ treatment is indicated: * $P < 0.01$, ** $P < 0.05$. For further details, see the Materials and methods section.

amylase release from secretory granules. However, arachidonic acid enhanced the fusogenic activity of membranes pretreated with PLA₂ (Figure 6). Although a stimulatory effect of arachidonic acid was observed with membranes pretreated with either low or medium PLA₂ activity, the effect was especially clear at low PLA₂ activity (0.055 unit/ml) because this PLA₂ alone at this activity did not show any significant effect. These results suggest that the presence of arachidonic acid itself during the pretreatment of the membranes is also important.

Effect of lysophosphatidylcholine on treatment of plasma membranes with PLA₂

We also examined the effects of lysophosphatidylcholine on PLA₂ treatment in order to investigate the role of lysophospholipids, which are another product of hydrolysis of membrane phospholipids by PLA₂, in developing the fusogenic activity of plasma membranes. Plasma membranes that had been pretreated with 10 μ M lysophosphatidylcholine exhibited fuso-

Figure 7 Effects of lysophosphatidylcholine treatment of plasma membranes in the absence or presence of non-esterified fatty acid or PLA₂ on fusion and amylase release

Plasma membranes were treated with 10 μ M lysophosphatidylcholine (Lyso) in the presence of 20 μ M Ca²⁺ in the absence or presence of 10 μ M arachidonic acid (AA) or linoleic acid (LA) or of PLA₂ (1.75 units/ml). Secretory granules were incubated with the plasma membranes at 37 °C for 3 min. The amounts of plasma membrane used for measurement of the fluorescence signal (□) and amylase release (▨) were 125 and 38.4 μ g of protein respectively. Values are means $\pm$ S.E.M. from four to seven and three to ten experiments on fluorescence intensity and amylase release respectively. Values significantly different at $P < 0.05$ from the control value (*), from the value with lysophosphatidylcholine alone (**) and from the value with PLA₂ alone (****) are shown. For further details, see the Materials and methods section.

genic activity (Figure 7). Lysophosphatidylcholine in the presence of non-esterified fatty acid showed no further effect (except in the presence of 10 μ M linoleic acid when there was a further increase in amylase release), but in the presence of arachidonic acid it enhanced the fusogenic activity of membranes pretreated with PLA₂ (Figure 7). These results suggest that lysophosphatidylcholine was involved in the induction of fusogenic activity.

Light- and electron-microscopic observations of secretory granules incubated with plasma membranes

Secretory granules were incubated with PLA₂-treated or untreated plasma membranes under the same conditions as for the above experiments and then the granules and membranes were precipitated by centrifugation. Light-microscopic observation revealed that the precipitate of granules and untreated membranes contained many granules and membranes, whereas the precipitate of granules and PLA₂-treated membranes contained scarcely any granules. Electron-microscopic observation also revealed abundant and few secretory granules in precipitates of untreated and PLA₂-treated membranes respectively. The granules appeared similar before and after incubation with untreated membranes. These observations suggest that the secretory granules lose their granular conformation and release their contents on fusion with PLA₂-treated membranes.

DISCUSSION

In the present study, a crude plasma-membrane fraction was prepared by the method of Schramm and Danon [49]. To examine the specificity of plasma membrane in the fusion process, plasma membrane was purified from a crude preparation as described by Watson et al. [53]. About 28-fold purification was achieved, as judged by the activity of ouabain-sensitive (Na⁺-K⁺)-ATPase (the activities of the crude and purified fractions were 0.98 ± 0.12 and 27.77 ± 0.69 nmol of P_i liberated/min per mg of protein respectively; $n = 3$). There was no significant

difference in the potency of the two preparations, i.e. the amount of membrane in terms of protein required to induce fusogenic activity was similar in both preparations. Thus it seems that the fusogenic activity of plasma membranes produced by PLA₂ treatment is a common feature of biomembranes as suggested by Karli et al. [44]. Therefore the localization of secretory granules and the action site of PLA₂ after elevation of the intracellular Ca²⁺ level by stimulation appear to be most important for the fusion process in intact cells.

We obtained the following evidence that amylase secretion from secretory granules was induced by fusion of plasma membranes with granules. First, on incubation of granules with PLA₂-treated membranes, but not untreated membranes, the increase in the signal indicating membrane fusion and in amylase secretion both occurred within 30 s. Second, increasing the amount of membrane added increased the intensity of the signal and amylase secretion. Third, increasing the PLA₂ activity used for membrane treatment increased both the signal intensity and amylase secretion. Fourth, the presence of EGTA, quinacrine or albumin during PLA₂ treatment of the membranes inhibited the effects of the membranes on both the signal and amylase secretion. Fifth, the presence of exogenous arachidonic acid and/or lysophosphatidylcholine during PLA₂ treatment enhanced the increases in signal intensity and amylase secretion.

Under our conditions, amylase secretion was almost maximal within 30 s (Table 1), but the fusion signal continued to increase slightly after this time (Figure 2). This discrepancy may indicate that the fusion process necessary for amylase secretion occurred quite rapidly with rapid dequenching of R18 followed by further diffusion of R18 into the plasma membranes, although we could not detect phenomena that occurred within the first 30 s for technical reasons. There have been several reports that suggest a role for PLA₂ in the secretory processes of endocrine and exocrine cells. For example, it has been demonstrated that the activators of PLA₂, melittin and mastoparan, respectively increase release of prolactin from bovine pituitary cells [36] and release of insulin from rat pancreatic acinar cells [38]. Quinacrine is reported to inhibit catecholamine secretion from adrenal chromaffin cells [29]. However, these studies did not show the step in which PLA₂ is involved. In the present study, untreated plasma membranes had no effect on amylase secretion or the fusion process. However, they gained fusogenic activity on treatment with PLA₂. Their fusogenic activity depended on the amount of PLA₂ used for their pretreatment. Ca²⁺ ions were essential for induction of this fusogenic activity. Furthermore, quinacrine, an inhibitor of PLA₂, inhibited induction of fusogenic activity of the membranes. These results indicated that PLA₂ induced the fusogenic activity of the membranes. In contrast with our findings, Mizuno et al. [54] and Lee et al. [55] reported that untreated plasma membranes induced amylase release from rat parotid secretory granules. Possibly endogenous PLA₂ affected plasma membranes under their experimental conditions but the exact reason for the discrepancy is not clear.

Arachidonic acid has been shown to increase catecholamine secretion from bovine adrenal chromaffin cells [27,28]. In the present study, plasma membranes liberated arachidonic acid and linoleic acid during PLA₂ treatment of the membranes (Figure 4). The presence of albumin, which adsorbs non-esterified fatty acids, during the treatment inhibited induction of the fusogenic activity (Figure 5). Furthermore, exogenous arachidonic acid added during pretreatment with PLA₂ enhanced the fusogenic activity of the membranes (Figure 6). These results suggest that both of the fatty acids released from membrane phospholipids by PLA₂ activity or a metabolite(s) of arachidonic acid were also important in induction of the fusogenic activity of the mem-

branes. However, as induction of fusogenic activity of the membranes by PLA₂ treatment was not affected by the presence of inhibitors of cyclo-oxygenase and lipoxygenase, arachidonic acid itself and not one of its metabolites is important in the development of the fusogenic activity. Plasma membranes treated with arachidonic acid or linoleic acid alone did not induce any significant amylase release. Another product of hydrolysis of membrane phospholipids by PLA₂, lysophospholipids, also seem to be important in induction of the fusogenic activity, as lysophosphatidylcholine made plasma membrane fusogenic. Therefore the following two factors seem to be essential for the development of the fusogenic activity of the membranes: a conformational change in the membrane phospholipids induced by PLA₂, possibly production of lysophospholipids, and the action on the membranes of arachidonic acid and/or linoleic acid liberated from the membranes.

The present study showed that Ca²⁺ ions are not necessary for the process of fusion of secretory granules with the membranes, but are necessary for induction of fusogenic activity of the membranes by PLA₂. However, the study did not clarify whether activation of PLA₂ occurs in intact acinar cells during exocytotic amylase secretion, but PLA₂ has been shown to be translocated from the cytosol to the membrane in a Ca²⁺-dependent manner [56,57]. Thus it is possible that *in vivo* an increase in the intracellular Ca²⁺ ion concentration induced by an agonist results in translocation of PLA₂ in an active form from the cytosol to the membrane, which is its substrate.

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