Ca²⁺-independent fusion of synaptic vesicles with phospholipase A₂-treated presynaptic membranes *in vitro*

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To clarify the mechanism of exocytosis in neurotransmitter release, the fusion of synaptic vesicles with presynaptic membranes prepared from rat brain synaptosomes and concomitant acetylcholine (ACh) release induced by fusion of them were studied *in vitro*. Fusion of the synaptic vesicles with presynaptic membranes was measured by a fluorescence-dequenching assay with octadecyl rhodamine B. Synaptic vesicles fused with presynaptic membranes which had been pretreated with porcine phospholipase A₂ (PLA₂) in the presence of 20 μ M Ca²⁺ and released ACh, whereas synaptic vesicles did not interact with non-pretreated membranes. The fusion followed by ACh release depended (i) on the activity of PLA₂ during the membrane pretreatment, (ii) on the amount of pretreated membrane and (iii) on the duration of the pretreatment. The presence of Ca²⁺ ions during the pretreatment was essential for inducing a

fusogenic activity of the membranes, but Ca²⁺ ions were not required for the fusion itself because the fusion experiment was carried out in the presence of 5 mM EGTA without added Ca²⁺. The presence of quinacrine, an antagonist of PLA₂, during the membrane pretreatment inhibited their fusogenic activity, suggesting the importance of activation of PLA₂. Presence of albumin during the pretreatment, which is an adsorbent of free fatty acids, also inhibited the fusogenic activity. Arachidonic acid, when added during the pretreatment, potentiated the fusogenic activity of the membrane. These findings suggest that the conformational change in the presynaptic membrane phospholipids induced by PLA₂ and the presence of arachidonic acid produced by PLA₂ are important in the process of fusion of synaptic vesicles with the presynaptic membranes of rat brain, and that the fusion process itself is independent of Ca²⁺.

INTRODUCTION

Many proteins which are involved in synaptic vesicle exocytosis have been identified (for reviews, see references [1–7]). These proteins are localized specifically to secretory vesicles, presynaptic plasma membrane, or the cytoskeleton or cytoplasm of the nerve terminal. Although there is no doubt that the proteins are important in translocation, docking and fusion, and exocytosis of vesicles in regulated exocytosis, the precise mechanism of fusion of synaptic vesicles with synaptic plasma membrane is not known. Many lines of interesting evidence, indicating a pivotal role for phospholipase A₂ (PLA₂) in the secretory mechanism in adrenal chromaffin cells, have been accumulated. Acetylcholine (ACh) increased the intracellular Ca²⁺ concentration and resulted in release of catecholamines together with free arachidonic acid [8,9]. Exogenous arachidonic acid increased catecholamine secretion from the cells [10,11]. Melittin and mastoparan, which were found in honey bee venom and wasp venom respectively and were reported to activate PLA₂ [12–14], stimulated secretion in many cell types [15–20]. Inhibitors of PLA₂ were reported to inhibit the release of arachidonic acid and catecholamine secretion [21]. Moreover, secretory granules cross-linked by annexin II, a member of the annexin family of Ca²⁺- and phospholipid-binding proteins, fuse to the plasma membranes in the presence of arachidonic acid [22]. 14-3-3 proteins, suggested as some of the soluble proteins involved in Ca²⁺-dependent exocytosis [23,24], have Ca²⁺-dependent PLA₂ activity [25].

In a cell-free model system, Izumi et al. [26] showed the involvement of PLA₂ in catecholamine secretion. Karli et al. [27] first reported direct evidence of fusion of chromaffin granules

with the plasma membrane by a method of fluorescence dequenching of the lipid marker octadecyl rhodamine B (R18), as described by Hoekstra et al. [28]. We also suggested, in a model system of rat parotid acinar cells, that 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 and that the fusion process itself is independent of Ca²⁺ [29].

On the other hand, there are a few reports which suggest a role for PLA₂ in neurotransmitter release in the nervous system. Brain synaptic vesicles had Ca²⁺-dependent PLA₂ and an increase in the enzyme activity resulted in vesicle-vesicle aggregation [30]. Evidence for a coupling between the activity of PLA₂ and the release of catecholamines from brain synaptosomes has also been provided [31,32].

In the present study, we extended our previous work on exocytosis in parotid acinar cells in a cell-free system to brain cholinergic neurons. The results demonstrated Ca²⁺-independent fusion of synaptic vesicles with synaptic plasma membranes induced by PLA₂ treatment of the membranes.

MATERIALS AND METHODS

Materials

R18 was purchased from Molecular Probes (Eugene, OR, U.S.A.). Porcine pancreatic PLA₂ was from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Physostigmine salicylate, potassium glutamate, quinacrine dihydrochloride, BSA, arachi-

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

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donic acid and indomethacin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Eicosatetraenoic acid (ETYA), EGTA, piperazine-*N,N'*-bis-(2-ethanesulphonic acid) (Pipes), Hepes, 2-(12-hydroxydodeca-5,10-dienyl)-3,5,6-trimethyl-1,4-benzoquinone (AA861) and acetonitrile were from Wako Pure Chemicals (Osaka, Japan) and sucrose was from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of analytical grade.

Preparation of synaptic vesicles and presynaptic membranes

Synaptic vesicles and presynaptic membranes of rat brain were prepared by the method of Whittaker et al. [33]. In brief, male Wistar rats (250–350 g) were decapitated and the whole brain (minus cerebellum) of each rat was quickly removed, weighed and cut into pieces, which were homogenized in 0.32 M sucrose containing 0.1 mM EDTA buffered with 5 mM Hepes at pH 7.4 using a glass-Teflon homogenizer, rotating at 600–700 rev./min with six up-and-down strokes. The 10% (w/v) brain homogenate was centrifuged at 1000 *g* for 10 min to remove tissue debris and nuclei as precipitate (P_1). The supernatant was centrifuged at 12000 *g* for 20 min to give the crude synaptosomal pellet (P_2). The P_2 pellet was washed once by resuspension in the medium used for homogenization and by recentrifugation under the same conditions. The washed P_2 pellet was disrupted by hypo-osmotic shock with a sufficient volume of ice-cold water. The disrupted P_2 suspension was centrifuged at 12000 *g* for 20 min to remove mitochondria and myelin fragments for further fractionation. The supernatant containing synaptic vesicles and synaptosomal membranes was layered on to discontinuous sucrose-density gradients consisting of 5 ml each of 1.2, 1.0, 0.8, 0.6 and 0.4 M sucrose in each tube for centrifugation. After ultracentrifugation at 53000 *g* for 2 h using a P28S rotor on a Hitachi CP 70G ultracentrifuge, the synaptic vesicle fraction (D fraction) and the synaptosomal membrane fraction (G fraction) were collected from the 0.4 M sucrose layer and the 0.8/1.0 M sucrose interface, respectively, by aspiration with an L-shaped Pasteur pipette. These fractions collected were diluted with Pipes buffer consisting of 20 mM Pipes, 139 mM potassium glutamate, 5 mM glucose, 5 mM EGTA, pH 7.2, to lower sucrose concentrations in the fractions and ultracentrifuged at 100000 *g* for 40 min to yield firm pellets. Synaptic vesicles and synaptosomal membranes were suspended in 0.5 ml/head of Pipes buffer containing 10 μ M physostigmine salicylate (which did not affect the membrane fusion assessed by R18 fluorescence dequenching) and Ca^{2+} /Pipes buffer containing 20 μ M free Ca^{2+} ions (the free Ca^{2+} concentration was adjusted with Ca^{2+} /EGTA), respectively. The synaptosomal membrane suspension prepared above was used as a presynaptic membrane preparation. A 100 μ l aliquot of each suspension was taken for protein determination by the method of Lowry et al. [34]. All procedures described above were carried out at 4 °C. For ACh release experiments the fresh synaptic vesicles were used immediately after preparation. Both bio-material suspensions were kept frozen at –80 °C for later use in membrane fusion experiments.

Treatment of presynaptic membrane with PLA_2

The frozen presynaptic membranes were thawed, diluted with Ca^{2+} /Pipes buffer to make a suspension of 200 μ g of protein/ml (final concentration) and then incubated with or without porcine pancreatic PLA_2 (usually at 7 units/ml at 37 °C for 30 min unless otherwise indicated). The samples were cooled quickly on ice after incubation, diluted with ice-cold Pipes buffer and centrifuged at 100000 *g* for 40 min. The pellets of PLA_2 -treated and untreated membranes were washed once by resuspending in

Pipes buffer and recentrifugation to remove the residual PLA_2 and drugs tested. The pellets were resuspended in Pipes buffer containing 10 μ M physostigmine at 200 μ g and 2000 μ g of protein/ml for membrane fusion and ACh release experiments, respectively. The suspensions were kept frozen at –80 °C until required for use.

Labelling of synaptic vesicles with the fluorescent probe R18

The suspension of synaptic vesicles prepared as described above was diluted with Pipes buffer to 250 μ g of protein/ml (final concentration) and incubated with 20 μ M R18 in centrifuge tubes for 15 min at room temperature in the dark for preparation of R18-labelled synaptic vesicles. After incubation the suspension of the labelled synaptic vesicles was diluted with ice-cold Pipes buffer and centrifuged at 100000 *g* for 40 min. The pellet of the labelled synaptic vesicles was washed once by resuspension and recentrifugation using new centrifuge tubes to remove residual R18. The washed pellet was resuspended in Pipes buffer containing 10 μ M physostigmine at 267 μ g of protein/ml and kept on ice in the dark until use.

Measurement of fusion of synaptic vesicles with presynaptic membranes

For the usual fusion assay 40 μ l of R18-labelled synaptic vesicle suspension (10.7 μ g of protein) was mixed with 1610 μ l of Pipes buffer containing 10 μ M physostigmine in a cuvette maintained at 30 °C by constant stirring with a small magnetic bar. The reaction was started by addition of 350 μ l (70 μ g of protein) of the pretreated presynaptic 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. The time course of increase in fluorescence intensity induced by dequenching on fusion of the synaptic vesicles with the presynaptic membranes was recorded on a continuous chart and values at 20 s intervals during automatic measurement. The values obtained were arbitrary ones inherent to the spectrofluorophotometer used. Therefore, we assessed the extent of membrane fusion in terms of the relative net increase in fluorescence for 5 min between different pretreatment groups of presynaptic membranes. The net increase in fluorescence induced by addition (70 μ g of protein/2 ml of total volume in a cuvette) of presynaptic membranes pretreated with 7 units/ml PLA_2 was represented as 100%. In preliminary experiments, this 100% fluorescence intensity corresponded to $11.9 \pm 1.5\%$ ($n = 10$) of the fluorescence obtained at an infinite dilution of R18 after addition of detergent (0.2% Triton X-100).

ACh release from synaptic vesicles

The reaction was started by addition of 0.5 ml of synaptic vesicle suspension (908 ± 52 μ g of protein, mean $\pm$ S.E.M., $n = 20$) to 0.5 ml of PLA_2 -treated or untreated presynaptic membranes (250, 500, 750 and 1000 μ g of protein) or 0.5 ml of Pipes buffer containing 10 μ M physostigmine without membranes in centrifuge tubes. The incubation was carried out at 30 °C for 5 min in Ca^{2+} -free conditions (note: Pipes buffer incubation medium contained 5 mM EGTA), unless otherwise indicated. The ACh release experiment was carried out at 30 °C instead of 37 °C to lessen spontaneous ACh release from synaptic vesicles on warming, and hence the fusion experiment was also done so. After incubation, the reaction was stopped by quick cooling and then immediate centrifugation at 100000 *g* for 30 min at 4 °C. Released ACh in the supernatant was assayed by HPLC.

For analysis by HPLC, ACh in the supernatant was precipitated with KI₃ in the presence of tetraethylammonium as a co-precipitant and 200 pmol of ethylhomocholine as an internal standard. The quaternary ammonium compounds precipitated were dissolved with about 1 ml of acetonitrile and the solution was passed through a Bio-Rad AG 1×8 anion-exchange resin column to trap I₃⁻ in the solution. The effluents were evaporated to dryness under reduced pressure and the dried samples were stored in a refrigerator until required for assay. At use, the dried samples were dissolved with 100 µl of water and filtered through a 0.45-µm-pore-sized filter and injected into the HPLC assay system for ACh determination.

ACh determination

ACh was determined by Yanaco reversed-phase HPLC, which was developed first by Potter et al. [35], using a post-column enzyme reactor instead of the enzyme-flow system of the original method. The procedures are as follows: (1) the separation of ACh, choline and ethylhomocholine (added as an internal standard) by the Yanaco ODS-A reversed-phase column (4.0 mm × 250 mm); (2) ACh hydrolysis and conversion of the resultant choline into H₂O₂ by an Eicom AC-Enzypak column loaded with acetylcholinesterase and choline oxidase; (3) electrochemical detection of the H₂O₂ using a Yanaco VMD-101A voltammetry detector equipped with a platinum electrode; and (4) recording of electric signals using a Hitachi D-2500 chromatointegrator. The mobile phase consisted of 0.01 M sodium acetate, buffered to pH 5.0 with 0.02 M citric acid, containing 1.2 mM tetramethylammonium and 1.5 mg/l sodium octanesulphonate. The solution for enzyme reaction, consisting of 0.2 M potassium phosphate buffer (pH 8.5), was infused by a second pump and joined with the effluent from the reversed-phase column at a point prior to the AC-Enzypak column so that the pH of the effluent could become suitable to the enzyme activities of AC-Enzypak. ACh was calculated by the internal standard method based on the height ratio of ACh peak versus the ethylhomocholine peak.

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 ACh by fusion of synaptic vesicles with presynaptic membranes

Synaptic vesicles prepared from rat brain contained 388 ± 16 pmol of ACh/mg of protein (mean $\pm$ S.E.M., *n* = 20) and released about $45.6 \pm 1.5\%$ (*n* = 20) of their total ACh on incubation at 30 °C for 5 min (spontaneous release). Without pretreatment, presynaptic membranes did not affect the release of ACh, but the membranes pretreated with PLA₂ in the presence of 20 µM Ca²⁺ increased the release. In particular, the membranes pretreated with PLA₂ at 7 units/ml showed a significant effect (Table 1). Therefore, presynaptic membranes were pretreated with PLA₂ at 7 units/ml in the subsequent experiments. Increasing the amount of the membranes for incubation with synaptic vesicles resulted in an increase in ACh release (Figure 1). A 1 mg sample of the membranes pretreated with PLA₂ at 7 units/ml released $89.6 \pm 1.4\%$ (*n* = 20) of their total ACh during an incubation at 30 °C for 5 min. In these ACh release experiments, incubation was carried out under Ca²⁺-free conditions in

Table 1 Effect of PLA₂ activity during PLA₂ pretreatment of presynaptic membranes on ACh release from synaptic vesicles

Presynaptic membranes (200 µg of protein/ml) were pretreated with various activities of PLA₂ in the presence of 20 µM Ca²⁺ at 37 °C for 30 min. Synaptic vesicles were incubated with the membranes (1 mg of protein) at 30 °C for 5 min. 'Buffer' indicates ACh release into incubation medium without the membranes. The synaptic vesicle suspension contained a small amount of ACh released during a suspending procedure of synaptic vesicle fraction: 'Initial' indicates the release before the incubation. ACh released for 5 min incubation period was expressed as pmol/mg of protein of synaptic vesicles. Values are means $\pm$ S.E.M. from four experiments. Statistical significance: **P* < 0.02 and ***P* < 0.05, significantly different from the value for the buffer by Student's paired *t*-test. For further details, see the Materials and methods section.

	ACh released (pmol/mg of protein of synaptic vesicles)
Initial	57.4 $\pm$ 10.0
Buffer	173.5 $\pm$ 19.6
PLA ₂ pretreatment (units/ml)	
1.4	197.8 $\pm$ 24.8
3.5	236.2 $\pm$ 33.2*
7.0	400.0 $\pm$ 39.4*

Figure 1 Effect of the amounts of PLA₂-pretreated presynaptic membranes on ACh release from synaptic vesicles

Synaptic vesicles were incubated without (○) or with (●) various amounts of PLA₂-pretreated membranes at 30 °C for 5 min. Open square denotes initial value. Presynaptic membranes were pretreated with 7 units/ml PLA₂ in the presence of 20 µM Ca²⁺. For further details, see the Materials and methods section and the legend of Table 1. Values are means $\pm$ S.E.M. from 4–8 experiments.

contrast to incubation for pretreatment of the membranes with PLA₂. Incubation media for the ACh release contained 5 mM EGTA without added Ca²⁺.

Next, we examined fusion of synaptic vesicles with presynaptic membranes by a method of fluorescence dequenching of R18. Synaptic vesicles which had been preloaded with R18 were incubated with presynaptic membranes with or without PLA₂

Figure 2 Dependence of membrane fusion between R18-labelled synaptic vesicles and PLA₂-pretreated presynaptic membranes on the PLA₂ activities used (a), the pretreatment period (b), and the amounts of the PLA₂-pretreated membranes (c) and R18-labelled synaptic vesicles (d)

(a) Synaptic vesicles (10.7 µg of protein/incubation cuvette) which had been preloaded with the fluorescent dye R18 were incubated at 30 °C with presynaptic membranes (70 µg of protein/incubation cuvette, total volume 2 ml) pretreated without or with the various indicated activities of PLA₂ at 37 °C for 30 min. Abscissa indicates incubation time (in s) and at the time point of 200 s the presynaptic membrane suspension was poured into the cuvette. Ordinate indicates fluorescence increase in arbitrary units. Results shown in (a)–(d) are typical of two or three experiments. (b) The vesicles (10.7 µg of protein/incubation cuvette) were incubated with the membranes (70 µg of protein/incubation cuvette) pretreated with 7 units/ml PLA₂ at 37 °C for the various durations indicated. (c) The vesicles (10.7 µg/incubation cuvette) were incubated with indicated amounts of PLA₂-pretreated membranes. The membranes were pretreated with 7 units/ml PLA₂ at 37 °C for 30 min. (d) Indicated amounts of synaptic vesicles were incubated with the membranes (70 µg of protein/incubation cuvette) pretreated with 7 units/ml PLA₂ at 37 °C for 30 min.

Table 2 Effects on ACh release and fusion of PLA₂ pretreatment of presynaptic membranes in the presence of EGTA, quinacrine or albumin

Presynaptic membranes were pretreated without (–PLA₂) or with 7 units/ml PLA₂ in the presence of 20 µM Ca²⁺ (Control), 5 mM EGTA (Ca²⁺ free), 100 µM quinacrine or 100 µg/ml albumin. Released ACh during incubation of synaptic vesicles with the membranes (1 mg of protein) at 30 °C for 5 min was measured. Fusion of synaptic vesicles preloaded with R18 with the membranes was measured at 30 °C. Net increase in fluorescence intensity for 5 min in the control experiment was taken as 100%. Values are means ± S.E.M. from 3–10 and 4 experiments. Statistical significance: **P* < 0.025, significantly different from the value for the control by Student's non-paired *t*-test. For further details, see the legend of Table 1.

	ACh released (pmol/mg of synaptic vesicle protein)	Fluorescence increase (% of control)
Initial	42.5 ± 4.4 (10)	
Buffer	172.1 ± 6.0 (10)	
–PLA ₂	188.6 ± 9.6* (6)	3.2 ± 1.2 (4)
Control	317.1 ± 18.0 (10)	100
Ca ²⁺ free	188.6 ± 18.0* (3)	4.6 ± 1.3 (4)
Quinacrine	231.3 ± 32.0* (3)	39.6 ± 11.4 (4)
Albumin	220.2 ± 30.8* (3)	44.8 ± 10.3 (4)

pretreatment. Without pretreatment, the membranes did not affect the fluorescence signal (Figure 2a); however, the membranes treated with PLA₂ in the presence of 20 µM Ca²⁺ increased the fluorescence signal, indicating fusion of synaptic vesicles and membranes. The increase in fluorescence depended (i) on the concentration of PLA₂ used for the pretreatment of membranes (Figure 2a), (ii) on the duration of treatment with PLA₂ (Figure 2b), and (iii) on the amount of pretreated membranes for

incubation (Figure 2c). Increasing the amount of synaptic vesicles also increased the fluorescence (Figure 2d). Although PLA₂ pretreatment of the membranes was carried out in the presence of 20 µM Ca²⁺, as mentioned above, the fusion proceeded in Ca²⁺-free medium. Thus, the process of fusion of synaptic vesicles with presynaptic membranes followed by ACh release did not seem to require Ca²⁺.

Effects of EGTA, quinacrine and albumin on the pretreatment of presynaptic membranes with PLA₂

In the above experiments pretreatment of presynaptic membranes with PLA₂ was carried out in the presence of 20 µM Ca²⁺. Under Ca²⁺-free conditions PLA₂ pretreatment did not give fusogenic activity to the membranes: the presence of 5 mM EGTA during the pretreatment completely inhibited the stimulatory effects of the membranes on ACh release (Table 2) and fluorescence induction (Table 2). When quinacrine at 100 mM, which is an inhibitor of PLA₂, was added during the pretreatment, fusogenic activity of the membranes was significantly inhibited (Table 2). Addition of albumin, an adsorbent of free fatty acids, also significantly inhibited the ability of the membranes to become fusogenic (Table 2). From these results, it seems that non-esterified fatty acids liberated from the membranes by PLA₂ activity are important for fusion of synaptic vesicles and membranes, since albumin adsorbed non-esterified fatty acids [36].

Effects of indomethacin, ETYA and AA861 on pretreatment of the membranes with PLA₂

To test the possible involvement of metabolites of arachidonic acid in inducing fusogenic activity, effects of some inhibitors of arachidonic acid metabolism were examined. Indomethacin

Table 3 Effect on ACh release of PLA₂ pretreatment of presynaptic membranes in the presence of indomethacin, ETYA or AA861

Presynaptic membranes (200 µg of protein/ml) were pretreated with 7 units/ml PLA₂ in the presence of 5.5 µM indomethacin, 10 µM ETYA or 30 µM AA861 in the presence of 20 µM Ca²⁺. Synaptic vesicles were incubated with the membranes at 30 °C for 5 min. Values are means ± S.E.M. from three separate experiments. (NS) indicates values obtained with these inhibitors were not significantly different from the value with PLA₂ only (Control). For further details, see the Materials and methods section and the legend of Table 1.

	ACh released (pmol/mg of synaptic vesicle protein)
Initial	47.7 ± 1.4
Buffer	190.5 ± 15.6
Control	425.8 ± 28.8
Indomethacin	385.4 ± 17.6 (NS)
ETYA	414.5 ± 34.8 (NS)
AA861	514.0 ± 42.4 (NS)

Table 4 Effects on ACh release of PLA₂ pretreatment of presynaptic membranes in the presence of arachidonic acid

Presynaptic membranes (200 µg of protein/ml) were pretreated with 1.4, 3.5 or 7.0 units/ml PLA₂ in the absence or presence of 10 µM arachidonic acid. Evoked ACh release (total release minus spontaneous release, 'Buffer') from synaptic vesicles induced by PLA₂-pretreated membranes is shown. The membranes pretreated with 10 µM arachidonic acid alone in the absence of PLA₂ had no effect on the evoked ACh release from synaptic vesicles. Values are means ± S.E.M. from four separate experiments. Statistical significance: **P* < 0.05, significantly different from the value in the absence of arachidonic acid by Student's paired *t*-test.

Pretreatment	Evoked release of ACh (pmol/mg of synaptic vesicle protein)
PLA ₂ (units/ml)	
1.4	36.9 ± 8.3
1.4 + Arachidonic acid	119.5 ± 32.4*
3.5	85.1 ± 19.4
3.5 + Arachidonic acid	192.3 ± 47.3*
7.0	198.4 ± 47.2

Figure 3 Enhanced effects of PLA₂ pretreatment of presynaptic membranes in the presence of arachidonic acid on the membrane fusion between R18-labelled synaptic vesicles and the membranes

(a) Presynaptic membranes (200 µg of protein/ml) were pretreated without (— PLA₂) or with 7 units/ml PLA₂ in the absence or presence of various concentrations of arachidonic acid at 37 °C for 30 min. (b) Presynaptic membranes were pretreated with 1.4, 3.5 or 7.0 units/ml PLA₂ in the absence or presence of 10 µM arachidonic acid (A.A.). Synaptic vesicles preloaded with R18 were incubated at 30 °C with the membranes that were poured into the cuvette at the time point of 100 s. DMSO indicates 0.1% DMSO as vehicle control. The membranes pretreated with 10 µM arachidonic acid alone in the absence of PLA₂ did not induce the membrane fusion ('PLA₂ 0 + A.A.' line). Results shown are one of two separate experiments. For further details, see the Materials and methods section and the legend of Figure 2.

(5.6 µM), an inhibitor of cyclo-oxygenase, ETYA (10 µM), an inhibitor of 5- and 12-lipoxygenase, and AA861 (30 µM), an inhibitor of 5-lipoxygenase, all did not affect the ability of membranes to become fusogenic (Table 3). The findings, together with those on quinacrine- and albumin-treatment, suggest an important role of arachidonic acid itself, not its metabolites, in inducing fusogenic activity of the membranes.

Effects of arachidonic acid on pretreatment of presynaptic membranes with PLA₂

Presynaptic membranes which were pretreated with arachidonic acid (10 µM) in the absence of PLA₂ did not increase ACh release and fluorescence signal, indicating that arachidonic acid itself is ineffective (Figure 3a). When exogenous arachidonic acid was added together with PLA₂ during the pretreatment, however, it significantly enhanced the fusogenic activity of the membranes: arachidonic acid concentration-dependently increased the fluorescence signal (Figure 3a). Its effects on ACh release were more evident at lower activities of PLA₂ (1.4–3.5 units/ml), since at the highest activity of 7 units/ml ACh release was already close to maximum (Table 4). The results suggest that the presence of arachidonic acid during the pretreatment is also important in induction of the fusogenic activity of the membranes.

Time course of ACh release

Experimental results described in the preceding section suggest the intimate connection of ACh release with the membrane fusion between synaptic vesicles and the PLA₂-pretreated membranes. Thus we examined whether ACh release could have the same time course as the membrane fusion shown in Figures 2 and 3. The amount of ACh released during 0.5 min incubation had already reached the level obtained during 5 min incubation (Table 5), indicating that the ACh release should be evoked at an early stage within 0.5 min. On the other hand R18-fluorescence intensity, which meant the extent of membrane fusion, increased rapidly after the addition of PLA₂-pretreated membranes. Nevertheless, it did not reach the maximal level at 0.5 min and was at that time point still growing gradually. As far as 5 min duration was concerned, the time courses of the two events were different.

Fusion and ACh release in the presence of Ca²⁺

When incubation media contained 20 µM Ca²⁺ the fluorescence intensity almost reached the maximal level within 1 min after addition of the PLA₂-pretreated membranes and then levelled off, or sometimes slightly levelled down for the assay period. The

Table 5 Time course of ACh release from synaptic vesicles induced by PLA₂-pretreated presynaptic membranes

Presynaptic membranes (200 µg of protein/ml) were pretreated with 7 units/ml PLA₂ at 37 °C for 30 min. When synaptic vesicles were incubated with the membranes (1 mg of protein/ml) at 30 °C for the periods indicated in the Table, ACh was released from synaptic vesicles into Ca²⁺-free incubation medium, and then assayed with HPLC. 'Buffer' indicates ACh release for 30 s without the membranes; 'Initial' indicates the release before incubation. The amount of ACh released for the 5 min incubation period was taken as 100% (455 ± 14.7 pmol/mg of protein). Values are means ± S.E.M. from three separate experiments.

ACh released (%)	
Initial	14.7 ± 1.4
Buffer	27.8 ± 5.0
Incubation period (min)	
0.5	97.3 ± 3.2
1.0	100.9 ± 5.8
1.5	94.3 (2)
3.0	111.1 ± 4.4
5.0	100

increase in intensity at 1 min in the presence of 20 µM Ca²⁺ remained about two-thirds of that under Ca²⁺-free conditions. In contrast to fluorescence dequenching, evoked ACh release for a 1 min incubation in the presence of 20 µM Ca²⁺ was 112 ± 3% (mean ± S.E.M., *n* = 3) as compared with the release in the absence of Ca²⁺. It was found that ACh release and the membrane fusion monitored by fluorescence dequenching did not appear to proceed necessarily in parallel, especially at late stages of the fusion process.

DISCUSSION

The role of Ca²⁺ ions in regulated exocytosis has been studied intensely and their role as the trigger for exocytosis has been accepted widely. Many proteins which are specifically associated with synaptic vesicles or synaptic membranes were reported to be involved in the exocytotic process of synaptic vesicles (for reviews see [1,3]). Involvement of various cytoplasmic proteins in the process was also suggested [1–7,37,38]. Some of these proteins were suggested to be closely associated with Ca²⁺ channels or with the exocytotic process via its Ca²⁺-binding ability. Syntaxin, a synaptic membrane-associated protein, was suggested to make a complex with N-type Ca²⁺ channel protein [39,40]. Synaptotagmin [41–43] and rabphilin-3A [44], synaptic vesicle-associated proteins, were suggested to function as Ca²⁺ sensors. Synapsin I, a cytosolic Ca²⁺-binding protein, was suggested to participate in the translocation of synaptic vesicles to the active zone by receiving phosphorylation via a Ca²⁺/calmodulin-dependent process (for review see [3]). However, the precise mechanism of Ca²⁺-triggered exocytosis is still far from fully elucidated. In this study we examined which step in the release process requires Ca²⁺, priming or membrane fusion itself. It was the most noteworthy finding of the present study that the fusion process itself was Ca²⁺ independent, i.e. fusion occurred under Ca²⁺-free conditions. In contrast, Ca²⁺ was suggested to have an essential role in inducing fusogenic activity of the membrane via activation of PLA₂.

The difference between the two time courses of membrane fusion and ACh release might be interpreted as follows. The membrane fusion would start quickly at a point of contact between the synaptic vesicle membrane and the presynaptic membrane and then the fusion area would continue to enlarge over the wide range of the two membranes at a gradually slowing speed.

Full release of ACh from synaptic vesicles should occur at an early stage of the fusion process, possibly because of early formation of pores in the synaptic vesicle membrane due to the rapid dynamic change of membrane rheology. A late stage of fusion did not seem to have any relationship with ACh release. For the validity of this interpretation, further studies are needed, e.g. on real time monitoring of ACh release by the use of a chemiluminescence method for ACh detection and change of membrane fluidity. Increase in R18-fluorescence intensity, but not ACh release, was inhibited by the presence of 20 µM Ca²⁺. The cause could not be determined. The possibility that in the presence of a certain concentration of Ca²⁺ R18 fluorescence dequenching is unable to monitor real membrane fusion cannot be ruled out.

Pretreatment of the membranes with PLA₂ in the presence of albumin, an adsorbent of free fatty acids, significantly inhibited induction of fusogenic activity. However, the presence of inhibitors of arachidonic acid metabolism during the pretreatment did not affect it. Therefore, it seems that the presence of unesterified fatty acid liberated from the membranes during PLA₂ treatment was necessary for induction of fusogenic activity. The finding that the presence of exogenous arachidonic acid during the pretreatment of the membranes with PLA₂ potentiated the fusogenic activity of the membranes (Figure 3) supported this idea. These results are compatible with those on the secretory granules and plasma membranes of the parotid gland of rats [29], although every experiment which was carried out on the secretory granules could not be replicated in the present study due to limitation of the materials. As we suggested in the previous study, the following two factors seem to be essential for the induction of fusogenic activity of the membranes: a conformational change in the membrane phospholipids induced by PLA₂, and the action on the membranes of arachidonic acid and/or other unesterified fatty acid liberated from the membranes.

Presynaptic membranes were treated with exogenous porcine PLA₂ in the present study. Increase in ACh release and fluorescence is not due to a direct effect of PLA₂ on synaptic vesicles, since PLA₂-pretreated membranes were washed by centrifugation and, furthermore, incubation for fusion of presynaptic membranes with vesicles was carried out under Ca²⁺-free conditions under which PLA₂ was inactive. Furthermore a direct effect of arachidonic acid on the fusion process is unlikely, since arachidonic acid itself was not fusogenic when it was directly added into the fusion assay medium (results not shown).

To examine the specificity of the membranes in the fusion process, in addition to presynaptic membrane, plasma membranes of rat liver and kidney cells were pretreated with PLA₂ in a similar way. The resulting membranes also acquired fusogenic activity to the following extent: presynaptic membrane > liver cell membrane > kidney cell membrane (results not shown). Furthermore, synaptic vesicles or mitochondria also, when pretreated with PLA₂, led to fusion with R18-labelled synaptic vesicles to a similar extent to presynaptic membranes (results not shown). Therefore, fusogenic activity of plasma membranes produced by PLA₂ pretreatment seems to be a qualitatively common property in biomembranes, as suggested by Karli et al. [27] and by our previous results on the secretory granules and membranes [29].

The subcellular fractions that were prepared according to the method of Whittaker et al. [33] and used in this study were not necessarily pure. However, it is unlikely that contaminants alone, but not presynaptic membrane, contained in the membrane fraction contributed to fusion, since the contaminants should not distribute evenly in each fraction. The main component in any of synaptosomal membrane, synaptic vesicle and mitochondrial

fractions can be considered to contribute to fusion. In a cell-free system used in this study exogenous PLA₂ was added to a membrane suspension for induction of fusogenic activity. Therefore, contaminants were also able to contact PLA₂. However, in intact cells substrates for endogenous PLA₂ should be limited to membrane phospholipids, although the contribution of endogenous cytosolic 85 kDa PLA₂ to the *in vivo* neurotransmitter release system remains uncertain to date.

Exogenous porcine PLA₂ was used in the presence of Ca²⁺ in the present study. PLA₂ in a variety of cells is activated by numerous agonists whose receptors are coupled to GTP-binding proteins. The $\beta\gamma$ dimer of a GTP-binding protein was first suggested to activate PLA₂, but the mechanism by which a GTP-binding protein activates PLA₂ remained unclarified. Another PLA₂, a cytosolic 85 kDa protein identified in brain as well as in neutrophils, platelets, macrophage-like cells, liver and lung, was suggested to be involved in several signal transduction pathways [45]. This enzyme is activated by Ca²⁺, GTP-binding proteins and phosphorylation (for a review see [46]) with translocation to the plasma membrane where its substrate is located [47]. It seems likely that activation of this PLA₂ is involved in the modulation of neurotransmitter release. However, participation of other enzymes such as Ca²⁺-independent PLA₂ and lysosomal PLA₂ could not be excluded.

Unesterified fatty acids, especially arachidonic acid, liberated from membrane phospholipids by phospholipases were suggested to play an important role in cell signal transduction: effects of the fatty acids on phospholipase C, phospholipase D, adenylate cyclase, guanylate cyclase, several ion channels, GTP-binding proteins and protein kinases were extensively studied (for a review see [48]). In the present study, however, purified presynaptic membranes were pretreated with PLA₂ and then the membranes were incubated with purified synaptic vesicles which had been preloaded with fluorescent dye, R18. Therefore, a direct effect of arachidonic acid liberated by PLA₂ pretreatment or added exogenously on the conformation of presynaptic membrane phospholipids seems more probable. Phospholipids without esterified arachidonic acid in the *sn*-2 position and interaction of unesterified arachidonic acid with them might contribute to the conformational changes suggested in the present study.

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