γ-Secretase is an aspartic protease that hydrolyzes type I membrane proteins within the hydrophobic environment of the lipid bilayer. Using the CHAPSO-solubilized γ-secretase assay system, we previously found that γ-secretase activity was sensitive to the concentrations of detergent and phosphatidylcholine. This strongly suggests that the composition of the lipid bilayer has a significant impact on the activity of γ-secretase. Recently, level of secreted β-amylloid protein was reported to be attenuated by increasing levels of phosphatidylinositol 4,5-diphosphate (PI(4,5)P2) in cultured cells. However, it is not clear whether PI(4,5)P2 has a direct effect on γ-secretase activity. In this study, we found that phosphoinositides directly inhibited CHAPSO-solubilized γ-secretase activity. Interestingly, neither phosphatidylinositol nor inositol triphosphate altered γ-secretase activity. PI(4,5)P2 was also found to inhibit γ-secretase activity in CHAPSO-insoluble membrane microdomains (rafts). Kinetic analysis of β-amylloid protein production in the presence of PI(4,5)P2 suggested a competitive inhibition. Even though phosphoinositides are minor phospholipids of the membrane, the concentration of PI(4,5)P2 within the intact membrane has been reported to be in the range of 4–8 mM. The presence of PI(4,5)P2-rich rafts in the membrane has been reported in a range of cell types. Furthermore, γ-secretase is enriched in rafts. Taking these data together, we propose that phosphoinositides potentially regulate γ-secretase activity by suppressing its association with the substrate.
observation points to the possibility that a change in the composition of the lipid bilayer could have a significant impact on the enzymatic activity of γ-secretase. The level of secreted Aβ from cultured cells was reported to be attenuated by increasing levels of phosphatidylinositol 4,5-diphosphate [PI(4,5)P2], one of the phosphoinositides (19). The phosphoinositides play pivotal roles in numerous biological processes, such as ion channel regulation, membrane trafficking, cell polarity, and actin rearrangement (20–25).

PI(4,5)P2 is estimated as 0.3–2.0% of the total cellular lipid. McLaughlin et al. (26) estimated that the intracellular concentration of PI(4,5)P2, if uniformly distributed inside the cell, was in the range of 2–30 μM, based on the dissociation constants between PH domains and PI(4,5)P2. Bunce et al. (27) reported that the concentration of PI(4,5)P2 was in the range of 32–159 μM in several cell species. Even though the phosphoinositides are a minor component of cellular lipids, one can assume that their concentrations in the two-dimensional intact membrane would be higher than that reported for the total three-dimensional cell volume. If the volume of the membrane is estimated as ~10–20% of the cell volume, the concentrations of PI(4,5)P2 in the membrane are at least five times higher than those in the total cell volume. The local concentration of PI(4,5)P2 at the inner leaflet of neutrophil membrane was reported to be ~5 mM in the steady state (28). Further, Sheetz et al. (29, 30) showed that the concentration of PI(4,5)P2 was 4–8 mM in a 50-Å area of the inner leaflet of erythrocyte membrane. In addition, a number of reports showed that PI(4,5)P2 localizes in the detergent-insoluble microdomains (rafts) of the membrane (31–33). It has been proposed that there is a spatially confined pool of PI(4,5)P2 in the membrane (34–37). Thus it is reasonable to consider that the concentration of PI(4,5)P2 in the microdomains of the membrane is much higher than previously thought. γ-Secretase is also enriched in lipid raft microdomains (38–40). It is likely that the phosphoinositides and γ-secretase localize in the same membrane microdomains.

Furthermore, phosphoinositides were widely known to be modulated by their concentrations in membrane by extracellular stimulus in physiological condition (41). Studies of several PH domains fused with green fluorescent protein revealed that concentrations of PI(3,4)P2 and PI(3,4,5)P3 were elevated after stimulus in Dictyostelium cells and neutrophils in vivo (21, 22, 42–44). In neutrophils, it has been reported that the local concentration of PI(3,4,5)P3 at the inner leaflet of the plasma membrane is 5 μM and that after extracellular stimulation it increases to 200 μM (28). The concentration of PI(3,4)P2 is estimated to increase from 10–20 to 100–200 μM upon stimulation (28). Winks et al. (45) reported that concentration of PI(4,5)P2 increased from 192–381 to 417–1153 μM after expression of PI5K in superior cervical ganglia. Those observations suggest that concentration of phosphoinositides can be modulated in physiological conditions. Recently, increasing phosphoinositide (PI(4,5)P2) levels alter Aβ production by cultured cells (19), implying a cross-talk between phosphoinositides and γ-secretase. Here we examined whether there are direct effects of phosphoinositides on γ-secretase in both CHAPSO-soluble and -insoluble states.

**EXPERIMENTAL PROCEDURES**

*Cell Culture—* Chinese hamster ovary (CHO) cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal bovine serum (Invitrogen) and penicillin/streptomycin (Invitrogen). Stable T-Rex-CHO cells (Invitrogen) inducibly expressing C99 were grown in F-12 nutrient mixture (Invitrogen) containing 10% fetal bovine serum (Invitrogen), penicillin/streptomycin, 250 μg/ml Zeocin (Invitrogen), and 10 μg/ml Blasticidin S (Invitrogen) (46).

*γ-Secretase Assay and Aβ Quantification—* Microsomal fractions of CHO cells were obtained as previously described (15) and solubilized on ice by the addition of equal volumes of 2× NK buffer (50 mM PIPES, pH 7.2, 250 mM sucrose, 1 mM EGTA, 2% CHAPSO, 1 mM diisopropyl phosphorofluoridate, 20 μg/ml antipain, 20 μg/ml leupeptin, 10 μg/ml TLCK, 10 mM phenanthrolone, and 2 mM thiorphan). The supernatant obtained after 100,000 × g centrifugation for 1 h was diluted with three volumes of the dilution buffer (50 mM PIPES, pH 7.2, 0.166% CHAPSO, 250 mM sucrose, 1 mM EGTA, 1 mM diisopropyl phosphorofluoridate, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml TLCK, 5 mM phenanthrolone, 1 mM thiorphan, 1.33 μM pepstatin A, and 0.133% phosphatidylcholine). The diluted supernatants contained a final concentration of 0.1% (equivalent to 1.3 mM) phosphatidylcholine and 0.375% CHAPSO unless otherwise indicated. Defined amounts of C99-FLAG substrate were incubated with the CHAPSO lysate at 37 °C for 4 h (15). We observed that the addition of 1 μM pepstatin A prevented γ-secretase-independent C99-FLAG cleavage. Incubated reaction mixtures were subjected to Western blotting for Aβ quantification, as described (14). After Aβ transfer, the nitrocellulose membrane was boiled for 5 min in the aluminum boiling apparatus (Can Do, Tokyo, Japan) for enhanced detection. Aβ on the membrane were visualized by an ECL system (GE Healthcare) using the well characterized monoclonal antibodies 82E1, BA27 (highly specific for the Aβ40 carboxyl terminus), and BC05 (raised against Aβ35–43, specific for the Aβ42 carboxyl terminus, but cross-reactive with CTFs and full-length APP), for assessing the total Aβ, Aβ40, and Aβ42, respectively (47).

*Preparation of Notch Substrate—* For assessment of γ-secretase-dependent Notch S3 cleavage in the presence of phosphoinositides, we expressed an artificial Notch substrate with shortened intracellular domain (designated as ΔE Notch-FLAG) in Sf9 cells (48). Isolated Notch substrate was incubated with the CHAPSO lysate at 37 °C for 4 h (15) (supplemental Fig. S1A). γ-Secretase-dependent S3 cleavage was visualized by detecting shortened Notch intracellular domain fused with FLAG tag (sNICD-FLAG) with ANTI-FLAG® M2 monoclonal antibody (Sigma) (supplemental Fig. S1B). We confirmed that ΔE Notch-FLAG was cleaved at the bona fide S3 cleavage site by γ-secretase (see supplemental Fig. S1, C and D).

*Isolation of CHAPSO-insoluble Rafts—* The CHAPSO-insoluble fraction was obtained as described previously (40). The T-Rex-CHO stable cell line was cultured in the presence of tetracycline to induce expression of C99 (46). Microsomal
Effect of \( \text{PI(4,5)P}_2 \) on \( \gamma \)-Secretase

200 \( \mu \)g/ml G418 (49). The cells were treated with phosphatidylinositol specific PLC inhibitor, edelfosin (Calbiochem) at a concentration of 15 \( \mu \)M in the absence of G418 for 6 h. Microsomal fraction of the cells were prepared as described previously (15). 500 \( \mu \)l of the microsomal fraction (2.5 mg/ml protein concentration) was mixed with 1 ml of MeOH:CHCl\(_3\):2(2:1) and centrifuged at 15,000 rpm for 5 min at 4 °C. Resultant pellet was mixed with the same solvent to complete neutral lipids extraction. 750 \( \mu \)l of MeOH:CHCl\(_3\):12 N HCl (40:80:1) was added to the pellet and mixed for extraction of acidic lipids. Supernatant was transferred to a new 1.5-ml tube and mixed with 250 \( \mu \)l of CHCl\(_3\) and 450 \( \mu \)l of 0.1 N HCl. After centrifugation, the organic phase was transferred to a new tube and dried up. The dried lipid sample was reconstituted with 80 \( \mu \)l of CHCl\(_3\); MeOH:H\(_2\)O (1:2:0.8) and spotted onto PI(4,5)P\(_2\) Mass Strip (Echelon). PI(4,5)P\(_2\) in extracted lipid sample was detected with PLC-\( \delta \)1 PH domain glutathione 5-transferase-tagged protein (Echelon). Microsomal fraction prepared from cells treated with edelfosin was incubated at 37 °C for 0.5 h in the presence of 15 \( \mu \)M edelfosin and subjected to Western blot to assess effect of edelfosin on \( \gamma \)-secretase production from isolated membrane (10, 13).

Phospholipids and Derivatives—

- \( \alpha \)-Phosphatidylcholine (PC) was purchased from Sigma, dissolved in 1% CHAPSO solution, and stored as such. \( \delta \)-myoinositol 1,4,5-triphosphate from Sigma was dissolved in water to 19.6 mM stock solution. PI C-16, phosphatidylinositol 3-phosphate C-16, phosphatidylinositol 4-phosphate C-16, phosphatidylinositol 5-phosphate C-16 (PI(5)P\(_2\)), phosphatidylinositol 3,4-diphosphate C-16 (PI(3,4)P\(_2\)), phosphatidylinositol 4,5-diphosphate C-16 (PI(4,5)P\(_2\)) and phosphatidylinositol 3,4,5-triphosphate C-16 (PI(3,4,5)P\(_3\)) from Cayman Chemical were dissolved in 0.25% CHAPSO and stored as 8.45 mM solutions. It is essential to avoid multiple freezing and thawing of these stock solutions. Stock solutions were repackaged into smaller vials and stored at \(-20 \)°C. Defined amounts of phospholipids and derivatives were mixed with \( \gamma \)-secretase reaction mixtures for incubation as above.

Immunoprecipitation—\( \gamma \)-Secretase complex was immunoprecipitated with anti-nicastrin polyclonal antibody (Sigma), as previously described (15). After thorough washing, the \( \gamma \)-secretase complex bound to protein A-Sepharose beads was incubated in
0.25% CHAPSO buffer (50 mM PIPES, pH 7.2, 250 mM sucrose, 1 mM EGTA, 0.25% CHAPSO, 2 mM disopropyl phosphorofluoridate, 20 μg/ml antipain, 20 μg/ml leupeptin, 20 μg/ml TLCK, 10 mM phenanthroline, 2 mM thiorphan, and 0.1% phosphatidylcholine) with C99-FLAG substrate at 37 °C for 4 h together with defined concentrations of PI(4,5)P₂. To evaluate inhibitory effects of PI(4,5)P₂ on the interaction between γ-secretase and C99-FLAG substrate, C99-FLAG prebound anti-FLAG M2 agarose beads (Sigma) were mixed with the CHAPSO-solubilized microsomal fraction of CHO cells and incubated at 4 °C overnight in the presence or absence of 0.845 mM PI(4,5)P₂. The agarose beads were washed three times and subjected to Western blotting to visualize γ-secretase components, including nicastrin, carboxyl-terminal fragment (CTF) of presenilin 1, and Aph-1. Presenilin 1 CTF, Aph-1, and Pen-2 were detected with anti-presenilin 1 CTF antiserum (a gift from Dr. Iwatsubo, University of Tokyo), anti-Aph1 polyclonal antibody (Covance), and anti-Pen-2 polyclonal antibody (50), respectively.

RESULTS

Effects of PI(4,5)P₂ on γ-Secretase—To demonstrate the effects of phosphoinositides on γ-secretase activity, a CHAPSO-solubilized γ-secretase assay was performed in the presence of various concentrations of PI(4,5)P₂ (15). The addition of PC to the CHAPSO-solubilized γ-secretase reaction mixture at increasing concentrations up to 1.3 mM (equivalent to 0.1%) enhanced the production of Aβ as described previously (15, 17, 18) (Fig. 1A), whereas increasing concentrations of PI(4,5)P₂ dramatically reduced Aβ production with an IC₅₀ of 141 μM (equivalent to 0.016%) (Fig. 1B). The inhibitory effect of PI(4,5)P₂ was observed even in the presence of 0.1% (1.3 mM) PC, with the IC₅₀ being 551 μM (equivalent to 0.06%) (Fig. 1C). We observed that PI(4,5)P₂ suppressed AICD and Aβ production in parallel (data not shown).

FIGURE 2. Inhibition of γ-secretase activity by PI(4,5)P₂. γ-Secretase complex was immunoprecipitated from CHAPSO-solubilized CHO membrane fraction with anti-nicastrin antibody. C99-FLAG substrate was incubated with the immunoprecipitated γ-secretase complex in the presence of PI(4,5)P₂. After 4 h of incubation at 37 °C, the reaction mixtures were subjected to Western blotting for Aβ quantification. Similar intensities of nicastrin signal were detected in all the samples (A, top panel). 82E1 revealed dose-dependent attenuation of Aβ production by PI(4,5)P₂ (A, bottom panel). The IC₅₀ was 0.0085% (equivalent to 72.3 μM). B, production of both Aβ40 and Aβ42 were suppressed in a similar fashion by increasing concentrations of PI(4,5)P₂. No significant differential effects on Aβ40/Aβ42 were noted. The data represent the means ± S.D. of three independent experiments.
Effect of PI(4,5)P₂ on γ-Secretase

FIGURE 3. Effects of phosphoinositides on Aβ and sNICD-FLAG productions. γ-Secretase substrates were incubated with CHAPSO-solubilized CHO membrane in the presence of various types of phosphoinositides. Aβ production was quantified as described (14, 15). A, PI and PI(5)P did not alter Aβ production; however, other phosphoinositides significantly attenuated Aβ production at a concentration of 0.85 mM. B, ΔE Notch-FLAG substrate was incubated with the CHAPSO-solubilized fraction. As seen in C99-FLAG, PI and PI(5)P failed to attenuate sNICD-FLAG production from ΔE Notch-FLAG. Interestingly, an increasing number of phosphate groups on the inositol ring tended to suppress γ-secretase activity to a greater extent. Inositol trisphosphate itself failed to alter Aβ production even at 1.69 mM (C). This indicates that phosphoinositols in combination with fatty acid is necessary to inhibit γ-secretase activity. The data represent the means ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01 (analysis of variance, Scheffe’s post hoc test compared with no treatment).

To further confirm a direct effect of PI(4,5)P₂ on γ-secretase activity, the γ-secretase complex that was immunoprecipitated with anti-nicastrin antibody was evaluated for Aβ production in the presence of PI(4,5)P₂. To enhance the detection of Aβ, the PC was kept to 0.1% in the reaction mixture. As shown in Fig. 2, PI(4,5)P₂ inhibited Aβ production in a dose-dependent manner, with the IC₅₀ being ~0.0085% (equivalent to 72.3 μM). The values of IC₅₀ in three experimental conditions were different from each other; however, those are in the range of physiological variance of phosphoinositides concentration reported (27). We considered that the concentrations of phosphoinositide employed in this study were physiologically relevant. Aβ40 and Aβ42 produced in the reaction mixture were quantified with BA27 and BC05, respectively. No significant differential effects on Aβ40 and Aβ42 production were noted (Fig. 2B).

In contrast to PC, PI(4,5)P₂ contains a large head group with two phosphates (26). We reasoned that this bulky head group caused inhibition of γ-secretase activity and tested various phosphoinositides using the CHAPSO-solubilized γ-secretase assay system. The addition of PI tended to reduce productions of Aβ and sNICD-FLAG; however, we could not detect statistic significance (Fig. 3, A and B, and supplemental Fig. S2). The addition of 0.85 mM phosphatidylinositol monophosphate (phosphatidylinositol 3-phosphate and phosphatidylinositol 4-phosphate) altered Aβ and sNICD-FLAG productions (Fig. 3, A and B, and supplemental Fig. S2). Interestingly, the addition of PI(5)P failed to alter Aβ and sNICD-FLAG productions significantly. Phosphatidylinositol diphosphates (PI(3,4)P₂ and PI(4,5)P₃) and phosphatidylinositol triphosphate (PI(3,4,5)P₃) showed significant inhibition of the enzyme activity, which implies that an increasing number of phosphate groups on the inositol ring enhances its inhibitory effect on γ-secretase, as well as the position of phosphate group on inositol ring (Fig. 3, A and B, and supplemental Fig. S2). It should be noted that these phosphoinositides inhibited AICD production in parallel with Aβ production (data not shown). However, inositol 1,4,5-triphosphate did not alter Aβ production in our assay system (Fig. 3C and supplemental Fig. S2). These data indicate that the phosphorylated inositol moiety in combination with a fatty acid is required for the inhibition of γ-secretase activity.

Inhibition by PI(4,5)P₂ of γ-Secretase Activity in CHAPSO-insoluble Rafts—We showed that phosphoinositides inhibited γ-secretase activity in the CHAPSO-soluble fraction. However, the inhibitory effects of phosphoinositides in the soluble fraction may not apply to γ-secretase that is embedded in membrane. Thus CHAPSO-insoluble rafts from CHO cell membranes were prepared by sucrose density gradient centrifugation and incubated with C99-FLAG substrate in the presence of PI(4,5)P₂. We found that lipid rafts prepared from CHO membranes produced Aβ from added C99-FLAG (supplemental Figs. S3 and S4). Fig. 4A shows decreased Aβ production from exogenous C99-FLAG by raft γ-secretase in the presence of 0.85 mM PI(4,5)P₂. This was also the case with endogenous C99 substrate (Fig. 4B). These data indicate that phosphoinositides suppress γ-secretase even in the membrane-embedded state.
Effect of PI(4,5)P² on γ-Secretase

**Fig. 4. Inhibition of Aβ production by CHAPSO-insoluble rafts in the presence of PI(4,5)P².** CHO cells were solubilized with 1% CHAPSO and subjected to sucrose gradient centrifugation to isolate a detergent-insoluble, floating fraction (see "Experimental Procedures" for details). Isolated CHAPSO-insoluble rafts were incubated with 100 nM C99-FLAG (exogenous) substrate in the presence of 0.85 mM PI(4,5)P² and Aβ production was quantified by the Western blotting. PI(4,5)P² was found to inhibit Aβ production (A). The rafts were prepared from CHO cells overexpressing C99 substrate. Isolated CHAPSO-insoluble fraction of the cells was incubated at 37 °C in the presence of 0.85 mM PI(4,5)P² (B). Aβ production from endogenous C99 was markedly affected by PI(4,5)P². The data represent the means ± S.D. of three independent experiments. *p < 0.005; **p < 0.001 (test compared with no treatment). 0' and 60' represent samples before and after 60 min of incubation at 37 °C.

**Effect of Phosphatidylinositol Specific PLC Inhibitor on Aβ Production from Isolated Membrane**—It was clearly shown that PI(4,5)P² affected γ-secretase activity in the detergent-soluble and membrane-embedded state. However, it is not known whether elevation of physiological PI(4,5)P² levels in cells alters Aβ production. Thus we tested whether pharmacological treatments that increase PI(4,5)P² level in cells would reproduce our findings on γ-secretase in the detergent-soluble states. Edelfosin is known as phosphatidylinositol-specific PLC inhibitor. Fig. 5A indicates increase of PI(4,5)P² level in isolated microsomal fraction of cells treated with edelfosin (see also Fig. 5B). As shown in Fig. 5 (C and F), edelfosin treatment decreased amount of secreted Aβ in the medium. We did not detect significant reduction of Aβ level in the isolated microsomal fraction of cells treated with edelfosin; however, de novo Aβ production from the isolated membrane after incubation at 37 °C for 30 min was significantly reduced (Fig. 5, C–E). These results support the idea that membrane lipid composition alters γ-secretase activity in cells.

**Effect of PI(4,5)P² on the Association between γ-Secretase Complex and C99-FLAG Substrate**—We showed inhibition by PI(4,5)P² of γ-secretase activity in both CHAPSO-soluble and -insoluble states. To gain further insight into the mechanisms of the inhibitory effects of phosphoinositides, we evaluated the association between γ-secretase complex and C99-FLAG in the presence of PI(4,5)P². Purified C99-FLAG, which was recaptured with M2 anti-FLAG agarose beads, was incubated with the CHAPSO-soluble fraction of CHO membranes at 4 °C overnight in the presence or absence of 0.85 mM PI(4,5)P². The C99-FLAG prebound agarose beads were spun down to evaluate the amounts of co-immunoprecipitated γ-secretase components. Substantial amounts of nicastrin, presenilin 1 CTF, Aph-1αL, and PEN-2 were detected bound to the M2 anti-FLAG agarose beads preincubated with C99-FLAG in the absence of PI(4,5)P² (Fig. 6A). In contrast, reduced amounts of these γ-secretase components were found in the presence of PI(4,5)P², but the interaction between the M2 anti-FLAG agarose beads and C99-FLAG was not affected by PI(4,5)P² (Fig. 6A, bottom panel). These data suggest that PI(4,5)P² interferes with the association between γ-secretase components and C99-FLAG. Interestingly, PI(4,5)P² markedly decreased the amount of Aph-1αL co-immunoprecipitated with C99-FLAG and concomitantly increased the level of Aph-1αL in the supernatant (Fig. 6A). This suggests that the association of Aph-1αL with C99-FLAG is more susceptible to phosphoinositol inhibition than that of other γ-secretase components. We also tested whether PI(4,5)P² peeled away γ-secretase from prebound C99-FLAG complex. As described above, purified C99-FLAG was recaptured with the M2 anti-FLAG agarose beads and then incubated with CHAPSO-soluble fraction of the CHO membrane at 4 °C overnight in the absence of PI(4,5)P². After sufficient washing, C99-FLAG beads complexed to γ-secretase was reincubated at 4 °C overnight in the presence or the absence of 0.85 mM PI(4,5)P². Western blot analyses revealed that PI(4,5)P² had no effect on stripping of γ-secretase from prebound C99-FLAG (Fig. 6B).

Co-immunoprecipitation analyses suggest that PI(4,5)P² inhibits the association between γ-secretase complex and C99-FLAG (Fig. 6A). However, it is possible that PI(4,5)P² mediates dissociation of the γ-secretase complex itself, rather than of the interaction between γ-secretase complex and C99-FLAG. To rule out this possibility, the effect of PI(4,5)P² on the stability of the γ-secretase complex was investigated. The CHAPSO-soluble fraction was subjected to immunoprecipitation with anti-nicastrin antibody in the presence or absence of PI(4,5)P² to isolate the γ-secretase complex. If PI(4,5)P² induces disassembly of the γ-secretase complex, its components other than nicastrin would not be co-immunoprecipitated. We did not detect a significant difference between the signals for presenilin 1 CTF, Aph-1αL, and Pen-2 in the presence or absence of PI(4,5)P², suggesting that PI(4,5)P² has no effect on the interaction between nicastrin and other components in our assay system (Fig. 6C). Overall, our data suggest that PI(4,5)P² mediates its inhibitory effect by suppression of the association of γ-secretase with substrate.

**Kinetics of the Inhibition of γ-Secretase in the Presence of PI(4,5)P²**—We showed that PI(4,5)P² inhibited activity of γ-secretase through inhibition of substrate binding. This observation suggests that PI(4,5)P² potentially functions as a
competitive inhibitor of \( \beta \)-secretase. Thus we tested whether the kinetics of A\( \beta \) production in the presence of PI(4,5)P\(_2\) showed a double reciprocal plot typical of competitive inhibition. Various concentrations of C99-FLAG were incubated with CHAPSO-solubilized fractions of CHO membrane and defined amounts of PI(4,5)P\(_2\). After incubation, the reaction mixtures were subjected to Western blotting (Fig. 7A). As shown in Fig. 7B, double reciprocal plots of \( \gamma \)-secretase activity in the presence of PI(4,5)P\(_2\) generally displayed a pattern characteristic of competitive inhibition. This indicates that PI(4,5)P\(_2\) acts principally as a competitive inhibitor of \( \gamma \)-secretase in this reaction system.

**DISCUSSION**

Phosphoinositides are minor components of the phospholipids in the biological membrane. Nevertheless, they are pivotal signaling molecules involved in a number of biological processes, such as ion channel regulation, vesicle trafficking, actin polymerization, and cell migration (20–25). Recently it has been reported that the turnover of PI(4,5)P\(_2\) is affected in cells expressing a familial AD mutant of presenilin 1 (19). It was also shown that the PI(4,5)P\(_2\) level was inversely correlated with the levels of A\( \beta \) produced by cultured cells (19). PLC is known to hydrolyze PI(4,5)P\(_2\), and PLC-\( \delta \) is abundant in neurons from AD brain (51). These observations suggest that phosphoinositides including PI(4,5)P\(_2\) potentially regulate A\( \beta \) production and that modulation of phosphoinositide levels could offer a therapeutic approach for AD (19). Because A\( \beta \) is produced in the hydrophobic environment surrounded by the lipid bilayer and its secretion is mediated by vesicular trafficking, it is reasonable to postulate that the composition of the lipid bilayer has an influence on A\( \beta \) production and secretion. In fact, the production of A\( \beta \) was altered by lipids in in vitro \( \gamma \)-secretase assay systems (15, 17, 18, 52). In the present study, we have shown that phosphoinositides exhibited inhibitory effects on A\( \beta \) production by the CHAPSO-solubilized membrane fraction of CHO cells. This was also the case with presenilin 1 mutants (M146L and M233T) (data not shown). Moreover, we have shown that phospho-
inositides inhibited the activity of immunoprecipitated γ-secretase in the CHAPSO-solubilized assay system. Thus phosphoinositides have a direct effect on the activity of solubilized γ-secretase. In addition, PI(4,5)P$_2$ suppressed Aβ production by CHAPSO-insoluble rafts. Thus inhibitory effects of phosphoinositides were exerted not only on γ-secretase in the soluble state but also on γ-secretase embedded in the membrane.

![Figure 6](image)

**FIGURE 6.** PI(4,5)P$_2$ inhibits the association of γ-secretase with C99-FLAG substrate. Purified C99-FLAG that was recaptured with anti-FLAG agarose beads was incubated with CHAPSO-solubilized membrane fraction of CHO cells at 4 °C in the presence or absence of 0.85 mM PI(4,5)P$_2$. After thorough washing, the beads were subjected to Western blotting to visualize co-immunoprecipitated components. The agarose beads incubated in the presence of 0.85 mM PI(4,5)P$_2$ showed a significant decrease in the signals for nicastrin, presenilin 1 CTF, Aph-1aL and Pen-2 bands. The level of C99-FLAG bound to the beads did not alter in the presence of PI(4,5)P$_2$ (A, bottom panel). Interestingly, PI(4,5)P$_2$ failed to peel away γ-secretase complex prebound to C99-FLAG from the C99-FLAG substrate (B). To rule out the possibility of PI(4,5)P$_2$-induced disassembly of γ-secretase, the γ-secretase complex was immunoprecipitated with anti-nicastrin antibody in the presence or absence of 0.85 mM PI(4,5)P$_2$. The intensity of the co-immunoprecipitated γ-secretase components was not affected even in the presence of PI(4,5)P$_2$ (C). It should be noted that Aph-1aL exhibits longer migration distance in Tris/Tricine gel compared with that in Tris/glycine gel (A). These results suggest that γ-secretase complex is stable in the presence of PI(4,5)P$_2$ and that PI(4,5)P$_2$ inhibits the association between γ-secretase and the C99-FLAG substrate. The data are representative results of four independent experiments. sup and ppt indicate supernatant and precipitate, respectively, after immunoprecipitation.

γ-secretase and phosphoinositides in rafts.

As mentioned above, phosphoinositides inhibit Aβ production from C99-FLAG substrate by γ-secretase in both CHAPSO-soluble and -insoluble fractions. γ-Secretase cleaves not only APP but other type I membrane proteins after ectodomain shedding. It would be also important to evaluate inhibitory effects of phosphoinositides on cleavage of other substrates. We generated an artificial Notch sub-

PI(4,5)P$_2$ amounts to 0.3–2.0% of the total cellular lipid. The concentration of PI(4,5)P$_2$ was determined to be in a range of 32–159 μM in a hypothetical cell sphere (27). However, phosphoinositides are generally distributed in the two-dimensional membrane within the cell, not in the three-dimensional space within the cell body. Theoretically, the concentration of PI(4,5)P$_2$ in the lipid bilayer should be higher than that described above. It has been reported that the theoretical local concentration of PI(3,4,5)P$_3$ at the inner leaflet of the plasma membrane of neutrophils is 5 μM (28) and that after extracellular stimulation it increases to 200 μM. The concentration of PI(4,5)P$_2$ is estimated to increase from 10–20 to 100–200 μM upon stimulation. It has been reported that the concentration of PI(4,5)P$_2$ increases from 192–381 to 417–1153 μM after expression of PI5K in superior cervical ganglia (45). PI(4,5)P$_2$ is a major phosphatidylinositol diphosphate, and its local concentration was estimated to be 5 μM in the steady state level (28). Sheetz et al. (29, 30) determined PI(4,5)P$_2$ concentration to be in a range of 4–8 μM in intact erythrocyte membranes scaffolded by the spectrin network. Thus the concentrations of PI(4,5)P$_2$ employed in this study have considerable physiological relevance. In addition, PI(4,5)P$_2$ is reported to be concentrated at the inner leaflet of cholesterol-rich microdomains (rafts) (31–33). Furthermore, it has been reported that γ-secretase is also enriched in the rafts (38–40). We thus presumed that phosphoinositides including PI(4,5)P$_2$ and γ-secretase colocalize in the same microdomain in living cells. It would be important in future studies to examine the colocalization of PI(4,5)P$_2$ and γ-secretase in rafts.
of PI(4,5)P$_2$ the relative amount of Aph-1aL bound to the substrate was reduced compared with that of other components (Fig. 6A). Because we could not detect any difference in the amounts of co-immunoprecipitated Aph-1aL with anti-nicastrin antibody in the presence or absence of PI(4,5)P$_2$ (Fig. 6C), the reduction in Aph-1 bound to C99-FLAG may be accounted for by PI(4,5)P$_2$-sensitive direct association of Aph-1aL to the substrate. It implies that even free Aph-1aL (that does not participate in a γ-secretase complex) directly binds to C99-FLAG with PI(4,5)P$_2$ sensitivity. Kinetic analysis of Aβ production in the presence of PI(4,5)P$_2$ displayed a pattern consistent with competitive inhibition. This implies that phosphoinositides bind to substrate-binding site(s) of γ-secretase complex. To our knowledge, PI(4,5)P$_2$ is the first natural compound to competitively inhibit γ-secretase.

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REFERENCES
1. Brown, M. S., and Goldstein, J. L. (1997) Cell 89, 331–340
2. Brown, M. S., Ye, J., Rawson, R. B., and Goldstein, J. L. (2000) Cell 100, 391–398
3. Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 6382–6387
4. Lee, J. R., Urban, S., Garvey, C. F., and Freeman, M. (2001) Cell 107, 161–171
5. Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takashashi, Y., Thinkaran, G., and Iwatsubo, T. (2003) Nature 422, 438–441
6. Urban, S., Lee, J. R., and Freeman, M. (2001) Cell 107, 173–182
7. Weihofen, A., Birnbaum, M., Kirschner, M., Ashman, K., and Martoglio, B. (2002) Science 296, 2215–2218
8. Selkoe, D. J. (2001) Physiol. Rev. 81, 741–766
9. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendoza, E. A., Denis, P., Teplow, D. B., Ross, S., Amaranth, P., Loehr, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarsinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) Science 286, 735–741
10. Gu, Y., Misonou, H., Sato, T., Dohmae, N., Takio, K., and Ihara, Y. (2001) J. Biol. Chem. 276, 35235–35238
11. Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M. M., Teplow, D. B., and Haass, C. (2001) EMBO Rep. 2, 835–841
12. Weidemann, A., Eggert, S., Reinhard, F. B., Vogel, M., Paliga, K., Baier, G., Masters, C. L., Beureuther, K., and Evin, G. (2002) Biochemistry 41, 2825–2835

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13. Sato, T., Dohmae, N., Qi, Y., Kakuda, N., Misonou, H., Mitsumori, R., Maruyama, H., Koo, E. H., Haass, C., Takio, K., Morishima-Kawashima, M., Ishiura, S., and Ihara, Y. (2003) J. Biol. Chem. 278, 24294–24301
14. Funamoto, S., Morishima-Kawashima, M., Tanimura, Y., Hiotani, N., Saido, T. C., and Ihara, Y. (2004) Biochemistry 43, 13532–13540
15. Kakuda, N., Funamoto, S., Yagishita, S., Takami, M., Osawa, S., Dohmae, N., and Ihara, Y. (2006) J. Biol. Chem. 281, 14776–14786
16. Li, Y. M., Lai, M. T., Xu, M., Huang, Q., DiMuzio-Mower, J., Sardana, M. K., Shi, X. P., Yin, K. C., Shafer, J. A., and Gardell, S. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6138–6143
17. Fraering, P. C., Ye, W., Strub, J. M., Dolios, G., LaVoie, M. J., Otsawasiewski, B. L., van Dorselaer, A., Wang, R., Selkoe, D. J., and Wolfe, M. S. (2004) Biochemistry 43, 9774–9789
18. Fraering, P. C., LaVoie, M. J., Ye, W., Otsawasiewski, B. L., Kimberly, W. T., Selkoe, D. J., and Wolfe, M. S. (2004) Biochemistry 43, 323–333
19. Landman, N., Jeong, S. Y., Shin, S. Y., Voronov, S. V., Serban, G., Kang, M. S., Park, M. K., Di Paolo, G., Chung, S., and Kim, T. W. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 19524–19529
20. Brown, F. D., Rozelle, A. L., Yin, H. L., Balla, T., and Donaldson, J. G. (2001) J. Cell Biol. 154, 1007–1017
21. Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R. A. (2002) Cell 109, 611–629
22. Funamoto, S., Milan, K., Meili, R., and Firtel, R. A. (2001) J. Cell Biol. 153, 795–810
23. Kanzaki, M., Furukawa, M., Raab, W., and Pessin, J. E. (2004) J. Biol. Chem. 279, 30622–30633
24. Oliver, D., Lien, C. C., Baukrowitz, T., Jonas, P., and Fakler, B. (2004) Science 304, 265–270
25. Michailidis, I. E., Helton, T. D., Petrou, V. I., Mirshahi, T., Ehlers, M. D., and Logothetis, E. D. (2007) J. Neurosci. 27, 5523–5532
26. McLaughlin, S., Wang, J., Gambhir, A., and Murray, D. (2002) Annu. Rev. Biophys. Biomol. Struct. 31, 151–175
27. Bantse, C. M., French, P., Allen, P., Mountford, J. C., Moor, B., Greaves, M. F., Michell, R. H., and Brown, G. (1993) Biochem. J. 289, 667–673
28. Lemmon, M. A., and Ferguson, K. M. (2000) Biochem. J. 350, 1–18
29. Sheetz, M. P., Febrbroriello, P., and Koppel, D. E. (1982) Nature 296, 91–93
30. Sheetz, M. P. (2001) Nat. Rev. Mol. Cell Biol. 2, 392–396
31. Laux, T., Fukami, K., Thelen, M., Golub, T., Frey, D., and Caroni, P. (2000) J. Cell Biol. 149, 1455–1472
32. Rozelle, A. L., Machesky, L. M., Yamamoto, M., Driessens, M. H., Insall, R. H., Roth, M. G., Luby-Phelps, K., Marriott, G., Hall, A., and Yin, H. L. (2000) Curr. Biol. 10, 311–320
33. Golub, T., and Caroni, P. (2005) J. Cell Biol. 169, 151–165
34. Simonsen, A., Wurmser, A. E., Emir, S. D., and Stemmer, H. (2001) Curr. Opin. Cell Biol. 13, 485–492
35. Martin, T. F. (2001) Curr. Opin. Cell Biol. 13, 493–499
36. Hinchcliffe, K. A., Ciruela, A., and Irvine, R. F. (1998) Biochim. Biophys. Acta 1436, 87–104
37. Janney, P. A., and Lindberg, U. (2004) Nat. Rev. Mol. Cell Biol. 5, 658–666
38. Vetrivel, K. S., Cheng, H., Kim, S. H., Chen, Y., Barnes, N. Y., Parent, A. T., Sisodia, S. S., and Thinakaran, G. (2005) J. Biol. Chem. 280, 25892–25900
39. Vetrivel, K. S., Cheng, H., Lin, W., Sakurai, T., Li, T., Nukina, N., Wong, P. C. H., Xu, and Thinakaran, G. (2004) J. Biol. Chem. 279, 44945–44954
40. Wada, S., Morishima-Kawashima, M., Qi, Y., Misono, H., Shimada, Y., Ohno-Iwahashi, Y., and Ihara, Y. (2003) Biochemistry 42, 13977–13986
41. Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1991) Nature 351, 33–39
42. Wang, F., Herzmark, P., Weiner, O. D., Srinivasan, S., Servant, G., and Bourne, H. R. (2002) Nat. Cell Biol. 4, 513–518
43. Servant, G., Weiner, O. D., Herzmark, P., Balla, T., Sedat, J. W., and Bourne, H. R. (2000) Science 287, 1037–1040
44. Rickert, P., Weiner, O. D., Wang, F., Bourne, H. R., and Servant, G. (2000) Trends Cell Biol. 10, 466–473
45. Winks, J. S., Hughes, S., Filipпов, A. K., Tatulian, L., Abogadie, F. C., Brown, D. A., and Marsh, S. J. (2005) J. Neurosci. 25, 3400–3413
46. Qi-Takahara, Y., Morishima-Kawashima, M., Tanimura, Y., Dolios, G., Hiotani, N., Horikoshi, Y., Kametani, F., Maeda, M., Saiko, T. C., Wang, R., and Ihara, Y. (2005) J. Neurosci. 25, 436–445
47. Suzuki, N., Cheung, T. T., Cai, X. D., Otvos, L., Jr., Eckman, C., Golde, T. E., and Younkin, S. G. (1994) Science 264, 1336–1340
48. Saxena, M. T., Schroeter, E. H., Mumm, J. S., and Kopan, R. (2001) J. Biol. Chem. 276, 40268–40273
49. Koo, E. H., and Squazzo, S. L. (1994) J. Biol. Chem. 269, 17386–17389
50. Shimojo, M., Sahara, N., Murayama, M., Ichinose, H., and Takashima, A. (2007) Neurosci. Res. 57, 446–453
51. Shimohama, S., Homma, Y., Suenaga, T., Fujimoto, S., Taniguchi, T., Araki, W., Yamaoka, Y., Takenawa, T., and Kimura, J. (1991) Ann. J. Pathol. 139, 737–742
52. Wrigley, I. D., Schurov, I., Nunn, E. J., Martin, A. C., Clarke, E. E., Ellis, S., Bonnert, T. P., Shearman, M. S., and Beher, D. (2005) J. Biol. Chem. 280, 12523–12535
53. Dhe-Paganon, S., Ottinger, E. A., Nolte, R. T., Eck, M. J., and Shoeles, S. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8378–8383
54. Ferguson, K. M., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1994) Cell 79, 199–209
55. Ferguson, K. M., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1995) Cell 83, 1037–1046
56. Hyvonen, M., Macias, M. J., Nilges, M., Oshkinat, H., Saraste, M., and Wilmanns, M. (1995) EMBO J. 14, 4676–4685
57. Soisson, S. M., Nimnual, A. S., Uy, M., Bar-Sagi, D., and Kuriyan, J. (1998) Cell 95, 259–268