in cells expressing claudin-4 than in mock transfectant control cells (Fig. 5A). Similar results were obtained for MMP-9 and pro-MMP-9 (Fig. 5A). In contrast, expression of the other claudins (claudin-1, -2, -3, -15) did not affect so clearly the intensity of these bands (Fig. 5A). These results suggest that the expression of claudin-4 specifically increases MMP-2 and MMP-9 activity, and that this may be responsible for the claudin-4-mediated stimulation of cell invasion.

Finally, mRNA expression of *MMP-2* and *MMP-9* in cells expressing each claudin was examined by real-time RT-PCR. As shown in Fig. 5B, the mRNA expression of both genes was up-regulated in cells expressing claudin-4 but not in those expressing the other claudins, suggesting that the higher activity of MMP-2 and MMP-9 in claudin-4-expressing cells is at least partly due to their higher expression.

DISCUSSION

Although it is generally believed that an alteration in claudin expression is involved in tumorigenesis, the role of individual claudins in the regulation of cancer-related cell functions, such as invasion and migration and regulating the intercellular barrier function of TJs, has remained unclear. This is because various types of cells, some of which lack the ability to form functional TJs, have been used in different studies. Therefore, in this study, we systematically examined the effect of overexpression of various claudins on Caco-2 cell invasion and migration, as well as on the intercellular barrier function of TJs, thereby allowing all these parameters to be investigated in a single system.

Overexpression of claudin-4 or claudin-2 either increased or deceased, respectively, TER in Caco-2 cells, results that are consistent with those obtained in other types of cells.[27,40,41] Overexpression of claudin-4 or claudin-2 also decreased or increased, respectively, the paracellular permeability of FD4 in these cells, suggesting that these claudins can exert an effect on cancer development by modulating the accessibility of nutrients and growth factors. As each of these claudins localizes at TJs under our experimental conditions, their differing effects on TJ barrier function appear to be due to their differing activities at these sites rather than differences in localization.

Overexpression of claudin-4, but not the other claudins, stimulated the invasive activity of Caco-2 cells. A similar effect has been observed in ovarian cancer cells (HOSE), whereas the opposite effect was observed in pancreatic cancer cells (SUIT-2).[16,25] Despite stimulating cell invasion, overexpression of claudin-4 inhibited the migration of Caco-2 cells, although it specifically increased the expression and activity of MMP-2 and MMP-9. Thus, MMP activity rather than cell migration appears to represent the mode of action by which claudin-4 stimulates cell invasion. It is known that claudins affect cell physiology through recruiting signal transduction-related molecules at TJs.[42] Furthermore, claudin-1, -2, -3 and -5 have been suggested to recruit and activate pro-MMP-2.[26,43] However, since overexpressed claudin-4 exists ubiquitously in cells under our culture conditions, claudin-4 could be affecting the expression and activity of MMPs either directly or by modulating signal transduction in the cytoplasm. Supporting this notion, the co-localization of claudins with MMP-2 is not limited to TJs but is also observed in the cytoplasm.[43]

Overexpression of claudin-2 or claudin-3 and -4 stimulated or inhibited, respectively, the migration of Caco-2 cells. We also observed actin stress fibers in Caco-2 cells expressing claudin-2, and found that each of these claudins delocalized from the cell surface to intracellular compartments after wound formation (activation of migration activity), an event that has previously been reported only for claudin-3.[44] Thus, migration-stimulating signals induce delocalization of claudins into intracellular compartments, with some of these claudins having a positive effect on cell migration whereas some of others exert the opposite effect. At present, the mechanism by which the different claudins influence cell migration remains unclear.

In summary, the results of this study suggest that overexpression of claudin-2 stimulates cancer development by decreasing the intercellular barrier function of TJs and increasing cell migration. On the other hand, the overall effect of overexpression of claudin-4 on cancer development remains unclear, as it increases both the intercellular barrier function of TJs and cell invasion. Furthermore, we found that the subcellular localization of TJs differs between cells cultured at high density (cells contacting each other) and those grown at low density (migrating and growing cells). Thus, it seems that claudin expression affects the invasion and migration activities and the intercellular barrier function of TJs independently, with both effects being important for cancer development.

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Research Paper

Synthesis of Prostaglandin E_1 Phosphate Derivatives and Their Encapsulation in Biodegradable Nanoparticles

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Purpose. Prostaglandin E_1 (PGE_1) is an effective treatment for peripheral vascular diseases. The encapsulation of PGE_1 in nanoparticles for its sustained-release would improve its therapeutic effect and quality of life (QOL) of patients.
Methods. In order to encapsulate PGE_1 in nanoparticles prepared with a poly(lactide) homopolymer (PLA) and monomethoxy poly(ethyleneglycol)-PLA block copolymer (PEG-PLA), we synthesized a series of PGE_1 phosphate derivatives and tested their efficacy.
Results. Among them, PGE_1 2-(phosphonooxy)ethyl ester sodium salt (C2) showed the most efficient hydrolysis to yield PGE_1 in human serum. An *in vitro* platelet aggregation assay showed that C2 inhibited aggregation only after pre-incubation in serum, suggesting that C2 is a prodrug of PGE_1. *In vivo*, intravenous administration of C2 caused increase in cutaneous blood flow. In the presence of zinc ions, all of the synthesized PGE_1 phosphate derivatives could be encapsulated in PLA-nanoparticles. Use of L-PLA instead of D,L-PLA, and high molecular weight PLA resulted in a slower release of C2 from the nanoparticles.
Conclusions. We consider that C2-encapsulated nanoparticles prepared with L-PLA and PEG-D,L-PLA have good sustained-release profile of PGE_1, which is useful clinically.

KEY WORDS: biodegradable nanoparticles; encapsulation; prostaglandin E_1; zinc.

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ABBREVIATIONS: ADAM, 9-anthryldiazomethane; ADP, adenosine 5′-diphosphate; ALP, alkaline phosphatase; AS-013, Δ^8-9-*O*-butyryl prostaglandin F_1 butyl ester; $(BnO)_2$-PN$(CH(CH_3)_2)_2$, dibenzyl *N.N*-diisopropyl phosphoramidite; C*x* (x=2, 3, 4, 6, 12), PGE_1 *x*-(phosphonooxy)alkyl ester sodium salt; DEA, diethanolamine; DMAP, 4-dimethylaminopyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; EtOAc, ethyl acetate; FAB, fast atom bombardment; EPR, enhanced permeability and retention; HCl, hydrochloric acid; ^{1}H-NMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; *m*-CPBA, *m*-chloroperoxybenzoic acid; MPS, mononuclear phagocyte system; MQW, Milli-Q water; MS, mass spectra; Mw, molecular weight; PBS, phosphate-buffered saline; PEG, poly(ethyleneglycol)PGE1, prostaglandin E1; PLA, poly(lactide); PLE, porcine liver esterase; PPP, platelet-poor plasma; PRP, platelet-rich plasma; QOL, quality of life; S.E.M., standard error mean; Tris, tris(hydroxymethyl) aminomethane.

INTRODUCTION

The number of patients with peripheral obstructive vascular diseases such as arteriosclerosis obliterans has increased in line with aging of the population and increases in the prevalence of diabetes and hyperlipidemia. The condition can result in amputation of lower limbs or even death in severely affected patients (1). Various clinical treatments such as vascular bypass surgery have been developed for these diseases; however, the prognosis is not still good. Furthermore, a large number of patients (about 5–8% of elderly) suffer from mild peripheral vascular diseases (such as intermittent claudication) (2), for which effective drug treatments have not been established.

Prostaglandin E_1 (PGE_1), which has various physiological actions such as vasodilation, angiogenesis and inhibition of platelet aggregation, may thus serve as an effective treatment for peripheral obstructive vascular diseases. Results from a number of clinical and animal studies support this notion (3–5). However, the range of activities of PGE_1 are also related to adverse effects (such as hypotension and diarrhea) due to its distribution throughout the body when administered systemically (4,6). Furthermore, in addition to its chemical instability (hydrolysis to PGA_1), PGE_1 is easily inactivated by 15-hydroxydehydrogenase during the passage through the lung (7–9). Therefore, a drug delivery system that enables the stabilization of PGE_1 and its targeting at the site

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of vascular injury is important. With these points in mind, we developed lipo-PGE_1, a preparation incorporating PGE_1 into an oil-in-water lipid emulsion (lipid microspheres) consisting of a soybean oil core and lecithin surfactant with a diameter of approximately 200 nm (10–12). Incorporation of PGE_1 into lipid microspheres protects PGE_1 from inactivation in the lung and enables the selective delivery of PGE_1 to damaged blood vessels, resulting in enhanced therapeutic effects and reduced adverse effects (10,13,14). Lipo-PGE_1 is used clinically in Japan, South Korea and China for systemic administration and exhibits a more potent therapeutic effect for peripheral obstructive vascular diseases than does PGE_1 clathrated in cyclodextrin which is used clinically worldwide (12,15). We also synthesized a stable PGE_1 prodrug (Δ^8-9-*O*-butyryl prostaglandin F_1 butyl ester, AS-013) and lipo-AS-013 showed superior characteristics to lipo-PGE_1 in both animal and clinical studies (6,16,17). However, lipid microspheres cannot retain PGE_1 for a long period of time *in vivo* (16,18). Therefore, daily intravenous drip infusion is necessary for clinical treatment with lipo-PGE_1, which in turn requires patient hospitalization, resulting in a low quality of life (QOL). Encapsulation of PGE_1 in more stable nanoparticles that permit a longer-lasting therapeutic effect provided by the sustained-release of PGE_1 would consequently be of significant clinical benefit. Encapsulation of PGE_1 in nanoparticles with size of approximately 50–200 nm would enhance the selective delivery of PGE_1 to damaged blood vessels due to the enhanced permeability and retention (EPR) effect (14).

The encapsulation of drugs in biodegradable and biocompatible polymeric solid particles, such as poly(lactide) homopolymer (PLA)-particles is effective for achieving a sustained-release formulation of drugs (19–21). For example, encapsulation of luteinizing hormone-releasing hormone in microparticles prepared from PLA achieved a long-term therapeutic effect by enabling sustained-release of the hormone concomitant with the degradation of PLA, as has already been employed in clinical practice (22,23). Thus, PLA-nanoparticles (diameter 50–200 nm for the EPR effect) with sustained-release of PGE_1 may prove beneficial for the treatment of peripheral obstructive vascular diseases. One obstacle to the use of solid nanoparticles in the treatment of patients in clinical practice is the uptake of these particles by the mononuclear phagocyte system (MPS), or in other words by the reticuloendothelial system (21,24). Use of a monomethoxy poly (ethyleneglycol)-PLA block copolymer (PEG-PLA) enables the nanoparticles to escape from this uptake due to the steric barrier by which the PEG chain prevents interaction of the nanoparticles with opsonins and cells responsible for MPS, such as Kupffer cells (stealth effect) (18,21,24). Another obstacle is that relatively hydrophilic drugs, such as PGE_1 and betamethasone, are very hard to encapsulate in PLA-nanoparticles (25–27). Hydrophilic drugs can be encapsulated into nanoparticles using a double emulsion (*w/o/w*) process, however, the size of this type of particles is generally more than 400 nm diameter and may have less EPR effect. For betamethasone, we recently overcame this obstacle by using betamethasone phosphate. After insolubilization in the presence of zinc, betamethasone phosphate could be efficiently encapsulated in PLA-nanoparticles by the oil-in-water solvent diffusion method. Betamethasone phosphate released upon degradation of the nanoparticles could then be hydrolyzed to yield betamethasone both *in vitro* and *in vivo*, resulting in a long-lasting therapeutic effect (28,29).

In the present study, we synthesized a series of PGE_1 phosphate derivatives with different spacer (alkyl chain) length (PGE_1 *x*-(phosphonooxy)alkyl ester sodium salt (C*x*; x=2, 3, 4, 6, 12)) and evaluated their efficacy both *in vitro* and *in vivo*. All of these derivatives can be encapsulated in PLA-nanoparticles. Of the derivatives, C2 showed the most efficient hydrolysis to yield PGE_1 in human serum. C2 showed a potent inhibitory activity on platelet aggregation *in vitro* and increased cutaneous blood flow *in vivo*. C2-encapsulated nanoparticles prepared with L-PLA and PEG-D,L-PLA showed a good sustained-release profile of C2.

MATERIALS AND METHODS

Materials and Animals

D,L-PLA, zinc chloride and 1,4-dioxane were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). L-PLA was from Taki Chemical Co., Ltd. (Kakogawa, Japan). AS-013 was from our laboratory stock. PEG-D,L-PLA (average molecular weight of PEG and PLA are 5,600 and 9,400, respectively) was synthesized and evaluated as described previously (18,30). Porcine liver esterase (PLE) and human placenta alkaline phosphatase (ALP) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Wistar rats (6 weeks old, male) were from Kyudo Co., Ltd. (Kumamoto, Japan). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health, and were approved by the Animal Care Committee of Kumamoto University.

Analysis of Synthesized Molecules

Low-resolution- and high-resolution-fast atom bombardment (FAB) mass spectra (MS) were measured on a JMS-700 instrument (JEOL Ltd., Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JNM AL-300 instrument (300 MHz) (JEOL Ltd., Tokyo, Japan), using tetramethylsilane as an internal standard. Analytical thin-layer chromatography was performed using silica gel glass plates (60 F_{254}) (Merck Ltd., Tokyo, Japan). Column chromatography was performed using Silica gel 60N (Kantō Chemical Co., Tokyo, Japan). Compound 4 (PE1) shown in Fig. 1 was obtained from Daiichi Fine Chemical Co., Ltd. (Takaoka, Japan)

Synthesis of PGE_1 Phosphate

The structures of PGE_1 phosphate derivatives and outlines of their synthesis are shown in Fig. 1. The recovery of each compound and analysis of NMR data are provided in the supplementary information.

Compounds (1a–e) (1.7 mmol) were mixed with 1*H*-tetrazole (2.5 mmol) and dibenzyl *N,N*-diisopropyl phosphoramidite ($(BnO)_2$-$PN(CH(CH_3)_2)_2$) (3.4 mmol) in dichloromethane (10 ml). After stirring at room temperature overnight, *m*-chloroperoxybenzoic acid (*m*-CPBA) (3.4 mmol)

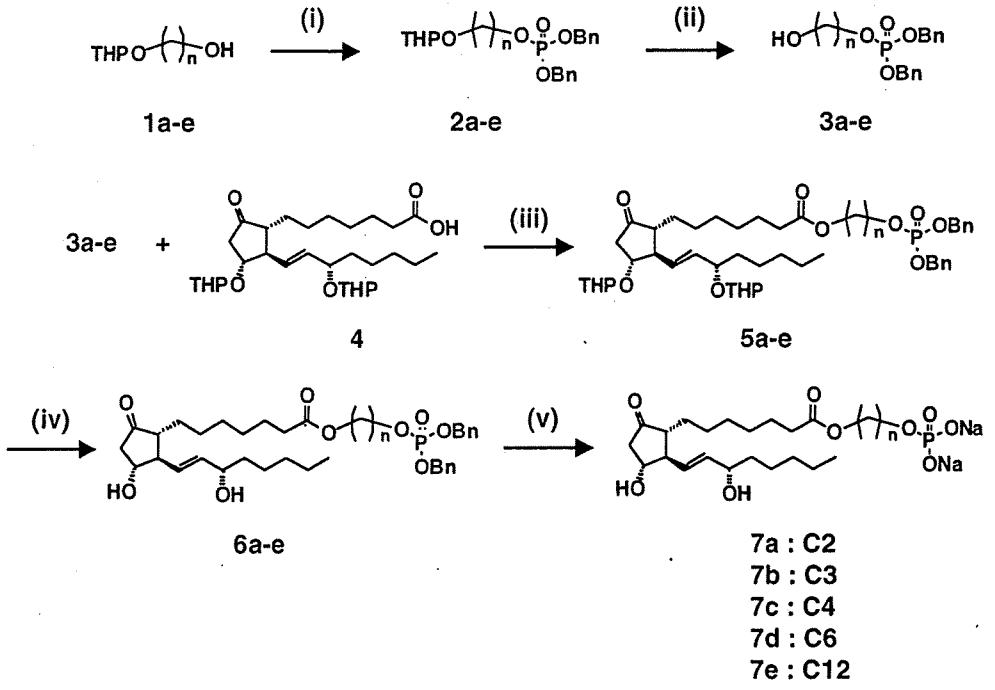

Fig. 1. Pathways for the synthesis of PGE_1 phosphate derivatives. Compounds: 1a–3a, 5a–7a (n=2); 1b–3b, 5b–7b (n=3); 1c–3c, 5c–7c (n=4); 1d–3d, 5d–7d (n=6); 1e–3e, 5e–7e (n=12). Reagents and solvents: *(i)* $(BnO)_2$-PN($CH(CH_3)_2)_2$, 1*H*-tetrazole, *m*-CPBA, dichloromethane; *(ii)* pyridinium *p*-toluene sulfonate, ethanol; *(iii)* EDC, DMAP, dichloromethane; *(iv)* acetic acid, tetrahydrofuran, MQW; *(v)* 1,4-cyclohexadiene, 10% palladium-carbon, acetic acid, sodium acetate, ethanol.

was added and stirred at room temperature for 30 min. The mixture was diluted with chloroform (30 ml), washed successively with saturated sodium hydrogen carbonate (10 ml×3) and saturated sodium chloride (10 ml×3). The organic layer was dried over sodium sulfate and concentrated in vacuo. The resulting residue was purified by silica gel column chromatography (from ethyl acetate (EtOAc)/hexane=1:1 to 100% EtOAc) to give compounds (2a–e) as a colourless oil.

Compounds (2a–e) (1.35 mmol) and pyridinium *p*-toluene sulfonate (0.3 mmol) in ethanol (5 ml) were stirred at 55°C for 3 h. After evaporation, the residue was purified by silica gel column chromatography (from EtOAc/hexane=3:2 to 100% EtOAc) to give compounds (3a–e) as a colourless oil.

Compounds (3a–e) (0.25 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (0.4 mmol), 4-dimethylaminopyridine (DMAP) (0.2 mmol) and compound 4 (PE1) (0.2 mmol) in dichloromethane (3 ml) were stirred at room temperature for 10 min. The mixture was diluted with chloroform (30 ml) and washed successively with saturated sodium hydrogen carbonate (10 ml×3) and saturated sodium chloride (10 ml×3). The organic layer was dried over sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane=1:1) to give compounds (5a–e) as a colourless oil.

Compounds (5a–e) (0.052 mmol) in acetic acid (1.8 ml)/tetrahydrofuran (0.45 ml)/Milli-Q water (MQW) (1.8 ml) were stirred at 35°C for 4 h and mixed with saturated sodium hydrogen carbonate (5 ml) at 0°C. The mixture was extracted with EtOAc (50 ml×3) and the combined organic layer was washed with saturated sodium chloride (10 ml×3) and dried over sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (from EtOAc/hexane=1:1 to 100% EtOAc) to give compounds (6a–e) as a colourless oil.

Compounds (6a–e) (0.026 mmol) were mixed with 10% palladium-carbon (64 mg) in 1,4-cyclohexadiene (2.8 ml)/acetic acid (0.2 ml)/ethanol (5 ml). After stirring at room temperature for 2 h, sodium acetate (0.052 mmol) was added and 10% palladium-carbon was removed by filtration, followed by washing with ethanol. The combined filtrate was concentrated to give compounds (7a–e) as a yellowish paste.

Determination of PGE_1 and Its Derivatives

A Waters Alliance system, running Empower software (Milford, MA), was used for the high-performance liquid chromatography (HPLC) analysis. Samples were separated using a 4.6×100-mm TSKgel Super-ODS column (Tosoh Co., Tokyo, Japan).

For detection of PGE_1 phosphate derivatives, solvent A (acetonitrile) and solvent B (5 mM ammonium acetate) were used at a flow rate of 0.5 ml/min. After injection of sample (0 min), the mobile phase was changed as follows; 25% solvent A (1 min), a linear gradient of 25–60% solvent A (7 min), a linear gradient of 60–100% solvent A (5 min) and 100% solvent A (7 min). The detection was performed at 195 nm.

For detection of PGE_1, solvent A (acetonitrile) and solvent B (MQW) were used at a flow rate of 0.3 ml/min. Samples were incubated with 9-anthryldiazomethane (ADAM) (Funakoshi Co. Ltd., Tokyo, Japan) at 37°C for 8 h. After injection of the sample (0 min), the mobile phase was changed as follows; 65% solvent A (25 min), a linear gradient of 65–100% solvent A

(10 min) and 100% solvent A (10 min). Fluorescence at 412 nm (fluorescence peak wavelength of ADAM reagent) was detected using a 2475 Multi λ Fluorescence Detector.

Preparation and Characterization of Nanoparticles

Nanoparticles were prepared by the oil-in-water solvent diffusion method as described previously (30). L-PLA in 1,4-dioxane or D,L-PLA in acetone was mixed with PEG-D,L-PLA and diethanolamine (DEA) in acetone and zinc chloride and each PGE_1 phosphate derivative in MQW (the total amount of block copolymers and homopolymer was fixed at 25 mg and total volume was 0.8 ml). Samples were incubated for 10 min at room temperature. The mixture was added dropwise (at a rate of 48 ml/h) to 25 ml of MQW stirred at 1,000 rpm. After addition of 0.5 ml of 0.5 M sodium citrate (pH 7.0) and 12.5 μl of 200 mg/ml Tween80, nanoparticles were purified and concentrated by ultrafiltration (Centriprep YM-50, Millipore Co., Billerica, MA).

For determination of the PGE_1 phosphate derivative content in nanoparticles, the nanoparticle suspension was mixed with 0.01 M sodium citrate (pH 7.0) and centrifuged at 50,000×*g* for 30 min. The pellet was washed and suspended in MQW, freeze-dried and weighed. The PGE_1 phosphate derivative content was determined using HPLC, as described above. The drug content was defined as the ratio of PGE_1 phosphate derivative weight to the total weight of nanoparticles.

Particle size and distribution were determined by the dynamic light scattering method (ZETASIZER Nano-ZS, Malvern Instruments Ltd., Malvern, UK) and the average diameter was calculated by Marquadt's method.

Treatment of PGE_1 Phosphate Derivatives with Serum, Plasma and Enzyme

This assay was performed as described in (16) with some modifications. The PGE_1 phosphate derivative (1 mM) was incubated at 37°C in 0.1 ml of human serum, rat plasma, or 0.1 M tris(hydroxymethyl) aminomethane (Tris)/hydrochloric acid (HCl) (pH 7.4) containing 2.5 U PLE or 25 mU ALP. Samples were taken periodically and diluted with ice-cold methanol. After incubation on ice for 30 min, the mixtures were centrifuged at 16,100×*g* for 10 min. The supernatants were evaporated to dryness and PGE_1 or its derivative content was determined by HPLC as described above.

Assay of Inhibition of Platelet Aggregation

This assay was performed as described in (17) with some modifications. Venous blood was collected from healthy human volunteers using 3.8% sodium citrate as an anticoagulant. Samples were centrifuged for 15 min at 160×*g* to obtain the upper phase (platelet-rich plasma (PRP)), and the lower phase was further centrifuged for 10 min at 1,500×*g* to obtain platelet-poor plasma (PPP). PRP was pre-incubated with PGE_1 or its derivatives and then mixed with adenosine 5′-diphosphate (ADP) (2 μM at final concentration). Samples were further incubated for 3 min and the extent of aggregation was measured using an NKK hematracer (PAC-8S, Niko Bioscience Co., Ltd., Tokyo, Japan). PPP was used as control.

Measurement of Cutaneous Blood Flow

This was performed as described in (17) with some modifications. Wistar rats were anaesthetized and a blood flow meter probe (ALF21, Advance Co., Osaka, Japan) was attached to left planta pedis. PGE_1 or C2 was intravenously administered via the tail vein at a dose of 10 nmol/kg.

RESULTS

The structures of PGE_1 phosphate derivatives and outlines of their synthesis are shown in Fig. 1. Tetrahydropyran ethers (1a–e) (31) were reacted with $(BnO)_2$-$PN(CH(CH_3)_2)_2$ in the presence of 1*H*-tetrazole to give dibenzyl phosphites, which were then oxidized with *m*-CPBA to yield dibenzyl phosphates (2a–e). The tetrahydropyran-protected group of 2a–e was deblocked by treatment with pyridinium *p*-toluene sulfonate in ethanol to afford alcohols (3a–e). These alcohols were then coupled to compound 4 (PE1), producing esters (5a–e). Removal of the tetrahydropyran-protected groups in 5a–e with aqueous acetic acid gave 6a–e. Catalytic hydrogenation with 1,4-cyclohexadiene of 6a–e was followed by treatment with sodium acetate to provide the desired sodium salts (7a–e).

We prepared Cn (n=2, 3, 4, 6, 12)-encapsulated nanoparticles with D,L-PLA, PEG-D,L-PLA, zinc chloride and DEA by the solvent diffusion method using the same protocol as that used for preparation of betamethasone phosphate-encapsulated PLA-nanoparticles (30). The particle size was similar for the different PGE_1 phosphate derivatives (Fig. 2A). On the other hand, the efficiency of encapsulation (drug content of nanoparticles) increased as a function of the spacer (alkyl chain) length (Fig. 2B). As was the case for betamethasone phosphate, very little of each PGE_1 phosphate derivative (less than 0.1%) could be encapsulated in the nanoparticles prepared in the absence of zinc chloride, and PGE_1 could not be encapsulated in the nanoparticles even in the presence of zinc chloride (data not shown), suggesting that insolubilization due to the interaction between zinc ion and phosphate group is important for efficient encapsulation. These PEG-containing nanoparticles seem to have a "core-corona" structure, because the zeta potential value was much lower than that of PEG-non-containing nanoparticles (deta not shown).

The efficiency of each PGE_1 phosphate derivative for hydrolysis by PLE or ALP was compared. As shown in Fig. 3A, in addition to AS-013, the compounds C6 and C12 were gradually hydrolyzed to yield PGE_1 in the presence of PLE, while C2, C3 and C4 were not. On the other hand, all of the PGE_1 phosphate derivatives tested were hydrolyzed by ALP, although the efficiency was different for each one (Fig. 3B). We also compared the production of PGE_1 from each PGE_1 phosphate derivative in human serum. As shown in Fig. 3C, a clear-cut production was observed only with AS-013 and C2. Based on results in Fig. 3A–C, we hypothesized that C2 can be hydrolyzed by esterase if the phosphate group is removed by phosphatase. To test this notion, we examined the production of PGE_1 from C2 in the presence of both PLE and ALP. The efficient production of PGE_1 was observed in the presence of both enzymes, but not with PLE or ALP alone (Fig. 3A, D). We also examined the production of PGE_1 from C2 in rat plasma and found that this took place

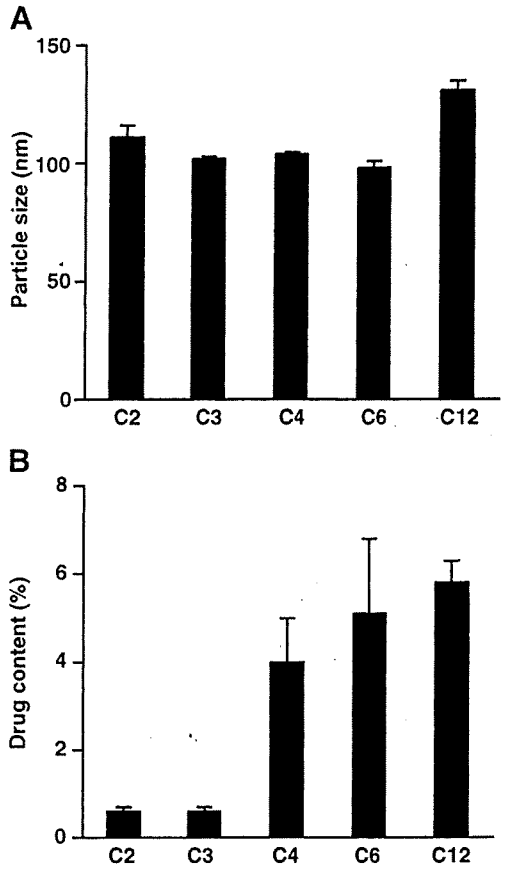

Fig. 2. Characterization of nanoparticles encapsulating PGE_1 phosphate derivatives. Nanoparticles encapsulating each PGE_1 phosphate derivative (5 mg) were prepared with 21 mg D,L-PLA and 4 mg PEG-D, L-PLA in the presence of 4.6 mg zinc chloride and 3.8 mg DEA by an oil-in-water solvent diffusion method. Particle size (**A**) and drug content (**B**) of the nanoparticles were determined as described in "MATERIALS AND METHODS." Values are mean ± S.E.M. (n=3).

within 30 min (Fig. 3E), which was more rapid than that in human serum (Fig. 3C). Based on the result in Fig. 3C, we selected C2 for further *in vitro* and *in vivo* analyses.

PGE_1 is chemically very unstable and the higher stability of AS-013 seems to be responsible for its relatively potent therapeutic effect (6,16,17). We compared the long-term chemical stability of C2 to that of AS-013 in water *in vitro*. As shown in Fig. 4A, C2 was more stable than AS-013 at 37° C. Stability at 4°C was indistinguishable between C2 and AS-013 (Fig. 4B).

We also examined the inhibitory effect of C2 on ADP-induced platelet aggregation *in vitro*, an established assay system for determining the anti-platelet aggregation activity of PGE_1 (16). We previously reported that, in contrast to PGE_1, pre-incubation in PRP is required for AS-013 to exert its inhibitory effect, because this step allows AS-013 to be hydrolyzed to PGE_1 (16). We reproduced this result here as shown in Table I. C2 showed no inhibitory effect on platelet aggregation without the pre-incubation step; however, it inhibited the aggregation as effectively as AS-013 and PGE_1 did after a 30 min pre-incubation (Table I). These results support the idea that C2 is efficiently hydrolyzed to yield PGE_1 in human blood.

We also evaluated the activity of C2 *in vivo* by monitoring its effect on cutaneous blood flow. Intravenous administration of PGE_1 induced a rapid and transient increase in blood flow (Fig. 5), as described previously (17). Compared to PGE_1, administration of C2 caused a slower-to-rise increase in blood flow, suggesting that C2 functions as a prodrug of PGE_1 and is gradually hydrolyzed in blood to yield PGE_1.

The *in vitro* release of C2 from different types of PLA-nanoparticles incubated at 37°C was examined in either phosphate-buffered saline (PBS) (Fig. 6C) or 50% fetal bovine serum (FBS) (Fig. 6D). Since the concentration of C2 in medium (released C2) was under the limitation of detection, the release was determined by measuring the C2 remaining in the particles. As shown in Fig. 6C, D, C2-encapsulated nanoparticles prepared with a blend of D,L-PLA (Mw=6,200) and PEG-D,L-PLA did not show good sustained-release profile in that more than half of encapsulated C2 was released from the nanoparticles within 6 h. We recently found that PLA-nanoparticles show better sustained-release profile when L-PLA is used instead of D,L-PLA (30), thus we applied this knowledge to the preparation of C2-encapsulated nanoparticles. As shown in Fig. 6C, D, C2-encapsulated nanoparticles prepared with L-PLA (Mw=5,500) released C2 slower than did those nanoparticles prepared using D,L-PLA (Mw=6,200). This modification of the PLA isomer did not clearly affect the particle size and the efficiency of C2 encapsulation (Fig. 6A, B).

As it has been suggested that higher molecular weight PLA results in nanoparticles with better sustained-release profile (32,33), we tested this idea for C2-encapsulated nanoparticles. The release of C2 from nanoparticles prepared with L-PLA (Mw=17,500) was much slower than that with L-PLA (Mw=5,500) (Fig. 6C, D). This modification of PLA molecular weight did not affect the particle size but did decrease the efficiency of C2 encapsulation (Fig. 6A, B). We determined the amount of Tween 80 in nanoparticles to about 6% of nanopartcles (*w/w*).

DISCUSSION

The incorporation of PGE_1 into lipid microspheres (lipo-PGE_1) that can then be administered via daily intravenous drip infusion is an effective means of treatment for patients with peripheral obstructive vascular diseases. However, daily drip infusion requires patient hospitalization, resulting in low QOL and prevents use of this drug for mild diseases, such as intermittent claudication. Thus, a new drug formulation that enables sustained-release of PGE_1 is therapeutically important. For this purpose, in this study, we have used the solvent diffusion method to prepare PGE_1 phosphate-encapsulated nanoparticles consisting of a blend of PEG-PLA and PLA. This idea is based on the following previous results: (1) PLA-nanoparticles slowly release the drug as the degradation of PLA proceeds (22,23,34); (2) a hydrophilic molecule with phosphate group (as in the case of betamethasone phosphate) becomes water-insoluble with zinc and can be encapsulated in PLA-nanoparticles (28,30); (3) incorporation of PEG-PLA in the PLA-nanoparticles prevents their uptake by MPS (18,21,24).

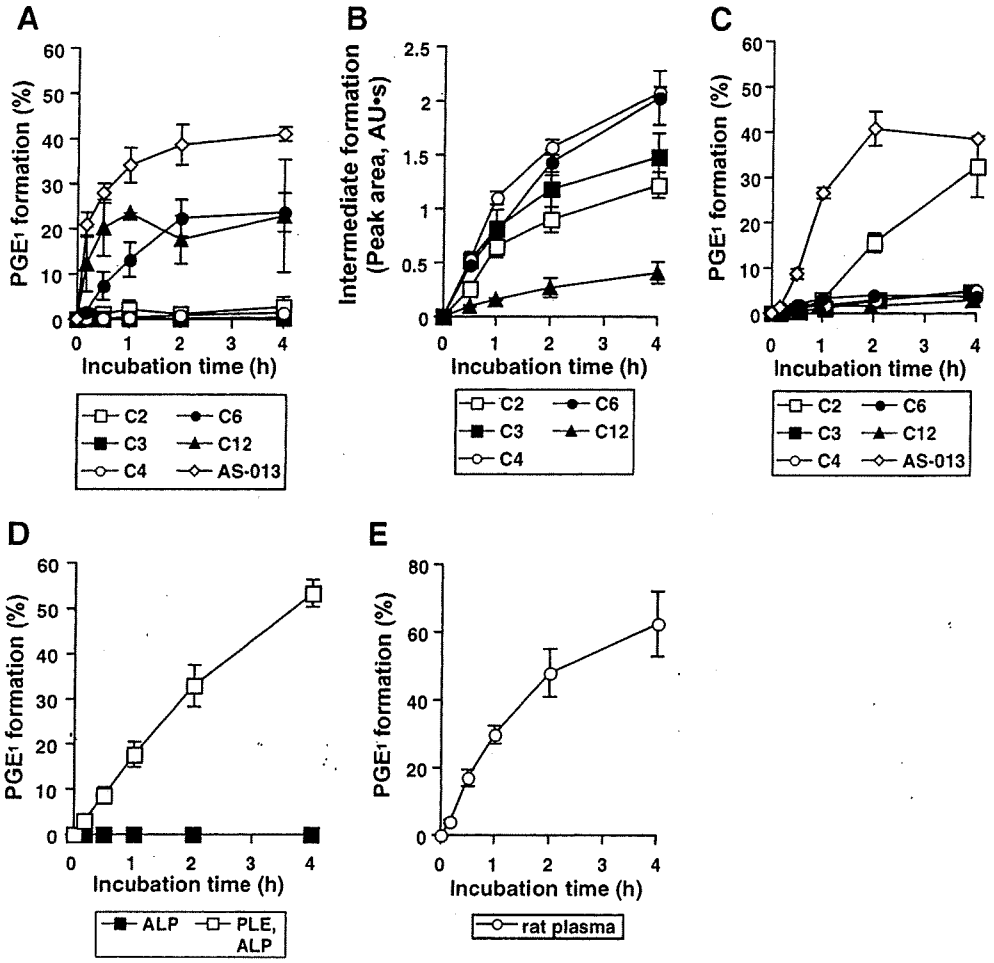

Fig. 3. Hydrolysis of PGE_1 phosphate derivatives. PGE_1 phosphate derivatives (**A–C**) or C2 only (**D**, **E**) were incubated at 37°C for indicated periods in the presence of PLE (**A**, **D**), ALP (**B**, **D**), human serum (**C**) or rat plasma (**E**). The amount of PGE_1 produced was determined by HPLC. The amount of each PGE_1 derivative before incubation was defined as the 100% value (**A**, **C–E**). The peak area corresponding to the dephosphorylated form of each derivative is shown in (**B**). Values are mean ± S.E.M. (n=3).

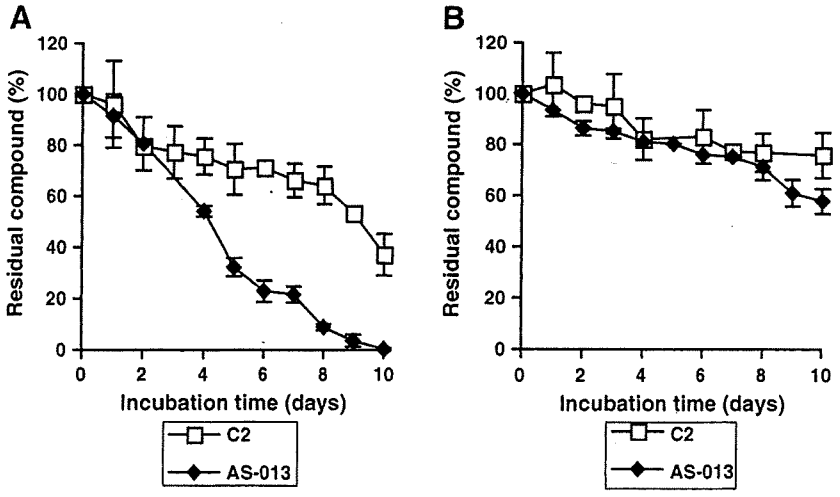

Fig. 4. Stability of C2 and AS-013. C2 or AS-013 (1 mM) was incubated at 37°C (**A**) or 4°C (**B**) in water for indicated periods. The amount of C2 or AS-013 remaining at specific time points was determined by HPLC. Values are mean ± S.E.M. (n=3).

Table I. Inhibitory Effect of PGE_1 and Its Derivatives on ADP-Induced Human Platelet Aggregation

Compound	ED50 (μM)		
	Incubation	0 min	15 min	30 min
PGE1		0.14±0.03	0.07±0.01	0.09±0.01
C2		>10	1.81±0.62	0.33±0.06
AS-013		4.92±1.11	0.81±0.06	0.73±0.07

Human PRP was pre-incubated with different concentrations of each PGE_1 derivative for the indicated periods at 37°C and platelet aggregation was induced by the addition of ADP. The concentration of each compound required for 50% inhibition of platelet aggregation (ED_{50}) is shown.

We synthesized a series of PGE_1 phosphate derivatives with different spacer (alkyl chain) length and established a protocol for their encapsulation in PLA-nanoparticles. The efficient encapsulation of each PGE_1 phosphate derivative in PLA-nanoparticles is dependent on the presence of zinc and DEA, i.e. zinc ion bridges between a carboxyl group of PLA and a phosphate group of the derivatives with a specific pH range obtained by DEA, seems to be important for the encapsulation process, as shown for the encapsulation of betamethasone phosphate (30). Among the derivatives, only C2 could be efficiently hydrolyzed to yield PGE_1 in human serum, showing that the spacer length is important for developing this type of prodrug. Surprisingly, C2 could be hydrolyzed to yield PGE_1 in the presence of PLE only after its dephosphorylation by ALP. The phosphate group of C2 may prevent esterase access to the ester bond, because PGE_1 phosphate derivatives with a longer spacer could be hydrolyzed in the presence of PLE. On the other hand, the more efficient hydrolysis of C2 than other derivatives in human serum suggests that de-phosphorylated C2 is better substrate for esterase in serum than that of other derivatives. Injectable nanoparticles that persist in the blood and contain other drugs could also offer a therapeutically important application

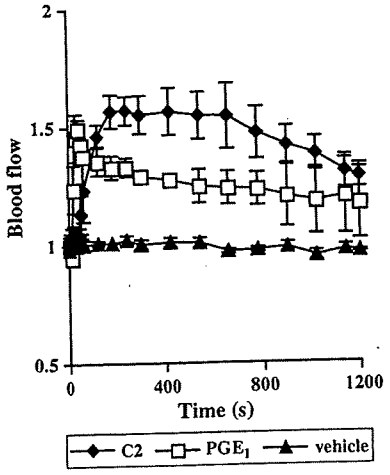

Fig. 5. Effect of C2 and PGE_1 on cutaneous blood flow. Wister rats were anaesthetized and C2, PGE_1 (10 nmol/kg) or vehicle was administered intravenously. The change of blood flow was monitored as a function of time. Values are mean ± S.E.M. (n=3).

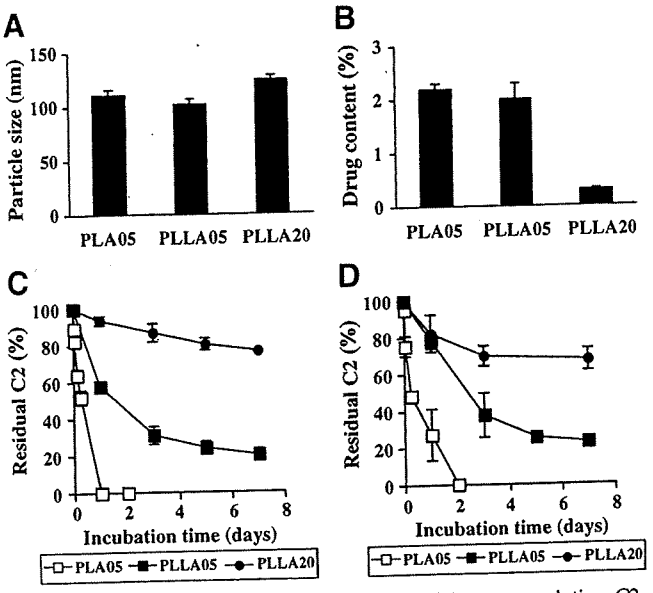

Fig. 6. Characterization of different nanoparticles encapsulating C2. Nanoparticles were prepared using D,L-PLA (Mw=6,200, 21 mg) (*PLA05*), L-PLA (Mw=5,500, 19 mg) (*PLLA05*) or L-PLA (Mw 17,500, 12.9 mg) (*PLLA20*) in the presence of zinc chloride (4.6, 2.7 or 2.7 mg for PLA05, PLLA05 or PLLA20, respectively) and DEA (1.9, 1.1 or 1.1 mg for PLA05, PLLA05 or PLLA20, respectively). Particle size (**A**) and drug content (**B**) were determined as described in the legend of Fig. 2. C2/PLA molar ratio in the formulation of PLA05, PLLA05 or PLLA20 was 0.16, 0.15 or 0.18, respectively. In (**C** and **D**), results are shown for experiments in which nanoparticles were dispersed in PBS (**C**) or 50% FBS (**D**) and incubated at 37°C for indicated periods. The C2 content remaining in the nanoparticles as a function of time was determined by HPLC. Values shown are mean ± S.E.M. (n=3).

because they enable site-specific and controlled-release delivery of the drug. The information obtained here for PGE_1 may be useful to develop similar approaches with respect to other prodrugs and their encapsulation in nanoparticles.

C2 showed good activity both *in vitro* and *in vivo*. The stability of C2 at 37°C in water was better than that of AS-013, which is much more stable than PGE_1 (16). In AS-013, the carbonyl group of PGE_1 at C-9 is acylated and a double bond between C-8 and C-9 is introduced to prevent the tautomerization (inactivation) of PGE_1 to its enol form (16,17). The phosphate group in C2 may stabilize PGE_1 in a different way. For example, acidic phosphate group in C2 may stabilize PGE_1 that is more stable under acidic conditions (35). Acylation at C-9 and the introduction of a double bond between C-8 and C-9 in C2 may result in better stability. An *in vitro* platelet aggregation assay showed that the activity of C2 is as potent as that of AS-013. Furthermore, the intravenous administration of C2 caused a slower-to-rise increase in blood flow, compared to PGE_1. This *in vivo* property may be due to the fact that C2 is more stable than PGE_1 and must be hydrolyzed to an active form. These results also point to the advantage of C2 not only as a molecule to be encapsulated in nanoparticles, but also for other clinical uses. For example, C2 in lipid microspheres (lipo-C2) may offer superior properties to those of lipo-PGE_1 and lipo-AS-013. We showed that the hydrolysis of C2 to yield PGE_1 took place more rapidly in rat plasma than in

human serum, even though it still took more than 30 min (Fig. 3E). On the other hand, blood flow increased very rapidly (within 5 min) after the intravenous administration of C2 (Fig. 5). This discrepancy was also observed for AS-013 (16) and may be due to the use of a very high concentration of C2 for the analysis in Fig. 3. The hydrolysis may occur rapidly *in vivo* where the drug concentration is much lower.

We achieved a good sustained-release profile of C2-encapsulated nanoparticles prepared using L-PLA, and in particular high molecular weight L-PLA. L-PLA has different physicochemical properties from D,L-PLA, such as thermodynamic stability and kinetic stability (36), which may be contribute to sustained-release profile of the nanoparticles. C2-containing nanoparticles prepared with L-PLA (Mw=17,500) were much better sustained-release profile than those prepared with L-PLA (Mw=5,500). Similar results have been reported for PLA-particles encapsulating other drugs (32,33).

Efficiency of C2 encapsulation in PLA-nanoparticles was very low. However, PGE_1 has high specific activities of vasodilation, angiogenesis and inhibition of platelet aggregation. For example, only 5 μg PGE_1 is clinically used per day for lipo-PGE_1. Therefore, even though the efficiency of C2 encapsulation is about 0.3% for nanoparticles prepared with L-PLA (Mw=17,500), the quantity of nanoparticles necessary to deliver an effective dose is calculated to be 16 mg of PLA-nanoparticle (50 μg of C2 corresponding to 35 (5 μg×7 days) μg of PGE_1) that is less than the amount of lipid microspheres (143 mg) administered with 5 μg PGE_1 as lipo-PGE_1.

We recently produced another type of PGE_1-encapsulated PLA-nanoparticles (18). In that report, PGE_1 itself was insolubilized with iron and encapsulated in PLA-nanoparticles. At present, we are not able to specify which formulation (PGE_1-iron or C2-zinc) would be more beneficial for clinical practice. The advantage of the PGE_1-iron formulation is that the safety and effectiveness of PGE_1 has already shown in clinical practice (C2 is a new compound and its safety needs to be tested more thoroughly). On the other hand, the advantage of the C2-zinc formulation is that C2 is more stable than PGE_1, which may result in a longer shelf life and easier mass production of the corresponding nanoparticles. Another advantage of the C2-zinc formulation is that the selective activation of C2 at sites of vascular injury and inflammation can be expected, since the alkaline phosphatase activity was suggested to be higher in peripheral tissues, particularly in inflamed tissue compared to blood (37,38). Furthermore, a number of beneficial activities of zinc, such as anti-atherosclerotic and anti-inflammatory activities, have been reported (39), which suggest that zinc in the C2-zinc PLA-nanoparticles may enhance the therapeutic effect of PGE_1. Further analyses of both formulations, especially of their pharmacological activity in animals, are necessary to determine which formulation is likely to be more suitable for clinical practice.

CONCLUSION

Based on results in this study, we consider that C2-encapsulated nanoparticles prepared with L-PLA and PEG-D, L-PLA have good sustained-release profile of PGE_1, which is useful clinically.

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Prostaglandin E_2 Stimulates the Production of Amyloid-β Peptides through Internalization of the EP_4 Receptor*[S]

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Amyloid-β (Aβ) peptides, generated by the proteolysis of β-amyloid precursor protein by β- and γ-secretases, play an important role in the pathogenesis of Alzheimer disease. Inflammation is also important. We recently reported that prostaglandin E_2 (PGE_2), a strong inducer of inflammation, stimulates the production of Aβ through EP_2 and EP_4 receptors, and here we have examined the molecular mechanism. Activation of EP_2 and EP_4 receptors is coupled to an increase in cellular cAMP levels and activation of protein kinase A (PKA). We found that inhibitors of adenylate cyclase and PKA suppress EP_2, but not EP_4, receptor-mediated stimulation of the Aβ production. In contrast, inhibitors of endocytosis suppressed EP_4, but not EP_2, receptor-mediated stimulation. Activation of γ-secretase was observed with the activation of EP_4 receptors but not EP_2 receptors. PGE_2-dependent internalization of the EP_4 receptor was observed, and cells expressing a mutant EP_4 receptor lacking the internalization activity did not exhibit PGE_2-stimulated production of Aβ. A physical interaction between the EP_4 receptor and PS-1, a catalytic subunit of γ-secretases, was revealed by immunoprecipitation assays. PGE_2-induced internalization of PS-1 and co-localization of EP_4, PS-1, and Rab7 (a marker of late endosomes and lysosomes) was observed. Co-localization of PS-1 and Rab7 was also observed in the brain of wild-type mice but not of EP_4 receptor null mice. These results suggest that PGE_2-stimulated production of Aβ involves EP_4 receptor-mediated endocytosis of PS-1 followed by activation of the γ-secretase, as well as EP_2 receptor-dependent activation of adenylate cyclase and PKA, both of which are important in the inflammation-mediated progression of Alzheimer disease.

Alzheimer disease (AD)[2] is the most common neurodegenerative disorder of the central nervous system and the leading cause of adult onset dementia. AD is characterized pathologically by the accumulation of tangles and senile plaques. Senile plaques are composed of the amyloid-β (Aβ) peptides Aβ40 and Aβ42 (1, 2). To generate Aβ, β-amyloid precursor protein (APP) is first cleaved by β-secretase and then by γ-secretase. Cleavage of APP by α-secretase produces non-amyloidogenic peptides (3, 4). The γ-secretase is an aspartyl protease complex composed of four core components, including presenilin (PS) 1 and PS2 (5). Early onset familial AD is linked to three genes, *APP*, *PS1*, and *PS2* (5, 6), strongly suggesting that γ-secretase-dependent production of Aβ is a key factor in the pathogenesis of AD. Therefore, cellular factors that affect the γ-secretase-dependent production of Aβ may be good targets for the development of drugs to prevent and treat AD.

Both APP and PS-1 are transmembrane proteins, and their intracellular localization is controlled by secretory and endocytic pathways. These proteins are modified in the endoplasmic reticulum and trafficked to the cell surface through the *trans*-Golgi network (TGN). Then, they are internalized again and trafficked to early endosomes. Next, they are trafficked to late endosomes and lysosomes (LEL), which are recycling endosomes that are targeted to the cell surface or the TGN (7–11). The production of Aβ seems to occur in a broad range of cellular compartments including the cell surface, TGN, and endosomes (12). Abnormalities of secretory and endocytic pathways have been observed in the brains of AD patients (9, 13). Importantly, factors that control these vesicle transport systems affect the production of Aβ. For example, overproduction of Rab5, a factor essential for traffic of vesicles to early endosomes, has been shown to stimulate the production of Aβ (14), and SorL1 has been shown to reduce the production of Aβ by stimulating the traffic of APP in early endosomes to the TGN (15, 16).

It has been suggested that inflammation is important in the pathogenesis of AD; chronic inflammation has been observed in the brains of AD patients, and trauma to the brain and ischemia, both of which can activate inflammation, are major risk factors for AD (17–19). Cyclooxygenase (COX) is essential for

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[2] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid-β; APP, β-amyloid precursor protein; CHO, Chinese hamster ovary; COX, cyclooxygenase; CTF, C-terminal fragment; DMEM, Dulbecco's modified Eagle's medium; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide; HA, hemagglutinin; HEK, human embryonic kidney; LEL, late endosomes and lysosomes; NTF, N-terminal fragment; pCPT-cAMP, 8-(4-chlorophenylthio)-cAMP; PKA, protein kinase A; PGE_2, prostaglandin E_2; PS, presenilin; siRNA, small interfering RNA; TGN, *trans*-Golgi network; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

the synthesis of prostaglandin E_2 (PGE_2), a potent inducer of inflammation and has two subtypes, COX-1 and COX-2. COX-1 is expressed constitutively, whereas expression of COX-2 is induced under inflammatory conditions and is responsible for the progression of inflammation (20–22). The following evidences of the involvement of PGE_2 (and COX-2) in the progression of AD suggest that they are good targets for the development of AD drugs: (i) Elevated levels of PGE_2 and overexpression of COX-2 have been observed in the brains of AD patients (23–25); (ii) the extent of COX-2 expression correlates with the amount of Aβ and the degree of progression of AD pathogenesis (26); (iii) transgenic mice constitutively overexpressing COX-2 show aging-dependent neural apoptosis and memory dysfunction (27); (iv) prolonged use of nonsteroidal anti-inflammatory drugs, inhibitors of COX, delays the onset and reduces the risk of AD (28); (v) PGE_2 stimulates the production of reactive oxygen species in microglia cells, resulting in activation of β-secretase (29).

We recently reported that PGE_2 stimulates the production of Aβ in human embryonic kidney (HEK) 293 and human neuroblastoma (SH-SY5Y) cells that stably express a form of APP with two mutations (K651N/M652L) (APPsw) that elevate cellular and secreted levels of Aβ (30). Similar results were reported by another group (31). Using agonists and antagonists specific for each of the four PGE_2 receptors (EP_1, EP_2, EP_3, and EP_4), we found that EP_4 receptors alone and also both EP_2 and EP_4 receptors are involved in PGE_2-stimulated production of Aβ in HEK293 or SH-SY5Y cells, respectively (30). Furthermore, experiments with transgenic mice suggest that EP_2 and EP_4 receptors are involved in the production of Aβ *in vivo* (30). Based on these results, we propose that antagonists of the EP_2 and/or EP_4 receptors may be therapeutically beneficial for the treatment of AD. Understanding the mechanism governing EP_2 and EP_4 receptor-mediated stimulation of production of Aβ by PGE_2 will be important for such drug development.

Activation of EP_2 and EP_4 receptors causes activation of adenylate cyclase and an increase in the cellular level of cAMP (32). We have shown that an EP_4 receptor agonist or both EP_2 and EP_4 receptor agonists increase the cellular level of cAMP in HEK293 or SH-SY5Y cells, respectively, and that a cAMP analogue, 8-(4-chlorophenylthio)-cAMP (pCPT-cAMP), increases the level of Aβ in HEK293 cells (30). These findings suggest that the cellular level of cAMP is important for PGE_2-stimulated production of Aβ. An increase in the cellular level of cAMP is known to activate protein kinase A (PKA), which is important for cAMP-regulated intracellular signal transduction (33). However, an inhibitor of PKA, N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide (H-89), does not block PGE_2-stimulated production of Aβ in HEK293 cells (30). Other cAMP-regulated signal transduction factors, such as phosphatidylinositol 3-kinase and Epac (exchange protein directly activated by cAMP), were also shown not to be involved in PGE_2-stimulated production of Aβ in HEK293 cells (30). Thus, the mechanism whereby the activation of EP_2 and EP_4 receptors stimulates the production of Aβ has remained unknown. In this study, by using inhibitors of adenylate cyclase and PKA, we found that activation of the EP_2 receptor stimulates production of Aβ through activation of adenylate cyclase and PKA. We also propose that activation of the EP_4 receptor causes its co-internalization with PS-1 (γ-secretase) into endosomes and that this co-internalization is important for EP_4 receptor-mediated stimulation of Aβ production by PGE_2 through the activation of γ-secretase.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM) and Ham-F12 medium were obtained from Nissui Pharmaceutical Co. The first-strand cDNA synthesis kit was from GE Healthcare. SQ22536 and fluorescent β-secretase substrate (H_2N-Arg-Glu-(EDANS)-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys-(DABCYL)-Arg-OH) were from Calbiochem. Lipofectamine (TM2000), Alexa Fluor 488 goat anti-rat immunoglobulin G, Alexa Fluor 594 goat anti-rabbit immunoglobulin G, and Alexa Fluor 488 goat anti-rabbit immunoglobulin G were purchased from Invitrogen. The plasmid pEGFP-N1 was obtained from Clontech. An antibody against actin was obtained from Santa Cruz Biotechnology. Fetal bovine serum, PGE_2, pCPT-cAMP, G418, H-89, concanavalin A, and antibodies against the C-terminal fragment (CTF) of APP, hemagglutinin (HA), Rab5, and Rab7 were from Sigma. An antibody against the N-terminal fragment (NTF) of PS-1 was from Chemicon or Immuno-Biological Laboratories, Inc., and the antibody against clathrin was from BD Biosciences. An antibody against EP_4 was from Cayman Chemical. The RNeasy kit and HiPerFect transfection reagent were from Qiagen. The APP-derived fluorescent substrate of γ-secretase (Nma-Gly-Gly-Val-Val-Ile-Ala-Thr-Val-Lys(Dnp)-D-Arg-D-Arg-D-Arg-NH_2) was from Peptide Institute Inc. Sulfo-NHS-S-S-biotin and UltraLink immobilized Neutravidin beads were from Pierce.

Animals—APP23 transgenic mice, a gift from Dr. M. Staufenbiel, were generated as described previously (34). APPsw/$EP_4^{-/-}$ and APPsw/$EP_4^{+/+}$ mice were generated as described previously (30). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health and were approved by the Animal Care Committee of Kumamoto University.

Cell Culture—HEK293, SH-SY5Y, and Chinese hamster ovary (CHO)-K1 cells were cultured in DMEM, DMEM/Ham-F12, and Ham-F12 medium, respectively, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C. HEK293 and SH-SY5Y cells expressing APPsw were from our laboratory stocks (35). CHO-K1 cells were from the RIKEN BioResource Center.

For transient expression, cells were seeded 24 h before transfection in 24-well plates at a density of 1.5×10^5 cells/well. Transfections were carried out using Lipofectamine (TM2000) according to the manufacturer's instructions. Cells were used for experiments after a 24-h recovery period. Transfection efficiency was determined in parallel plates by transfection of cells with pEGFP-N1 control vector. Transfection efficiencies were greater than 90% in all experiments. The stable transfectants

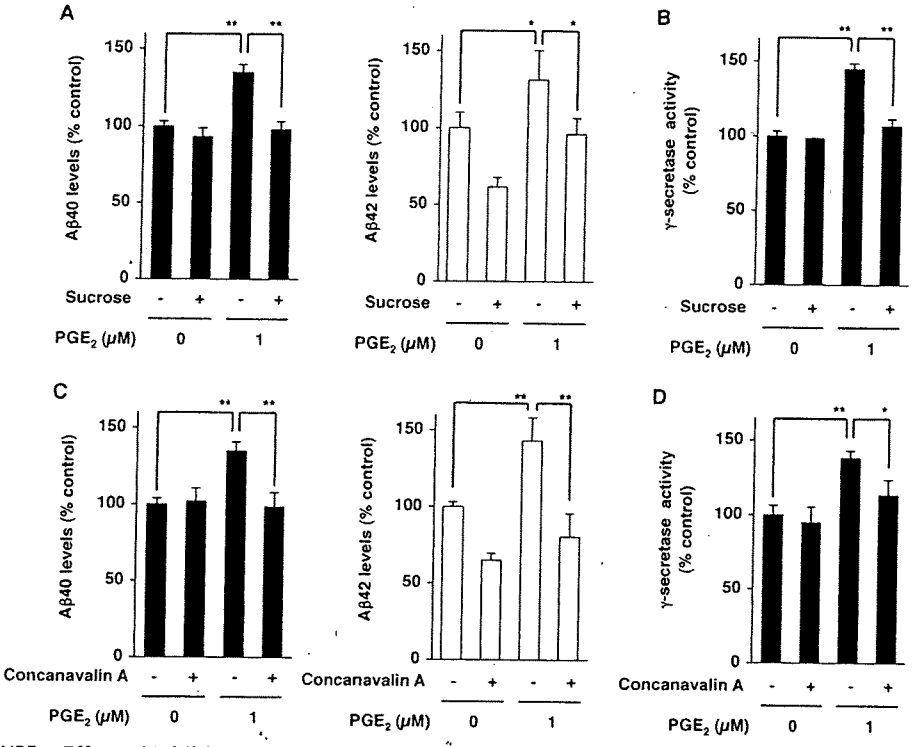

FIGURE 1. **Effect of inhibitors of endocytosis on PGE_2-stimulated production of Aβ in HEK293 cells.** HEK293 cells expressing APPsw were preincubated for 1 h with 0.5 M sucrose (*A* and *B*) or 0.25 mg/ml concanavalin A (*C* and *D*) and further incubated for 24 h with or without 1 μM PGE_2 in the presence of the same concentration of each inhibitor as in the preincubation step. The amounts of Aβ40 and Aβ42 in the conditioned medium were determined by sandwich enzyme-linked immunosorbent assay and expressed relative to the control (without PGE_2) (*A* and *C*). After incubation with or without PGE_2 for 1 h, membrane fractions were prepared and subjected to γ-secretase-mediated peptide cleavage assay as described under "Experimental Procedures" (*B* and *D*). Values are given as means ± S.D. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$.

expressing each gene were selected by immunoblotting or real-time reverse transcription-PCR analyses. Positive clones were maintained in the presence of 200 μg/ml G418.

Immunoblotting Analysis—Whole cell extracts were prepared as described previously (36). For detection of CTFα and CTFβ, membrane fractions were prepared as described previously (37). The protein concentration of each sample was determined by the Bradford method (38). Samples were applied to polyacrylamide-SDS gels (Tris-Tricine gel for the detection of CTFα and CTFβ or Tris-glycine gel for other proteins) and subjected to electrophoresis, after which proteins were immunoblotted with each antibody.

Sandwich Enzyme-linked Immunosorbent Assay for Aβ and γ-Secretase- or β-Secretase-mediated Peptide Cleavage Assay—Cells were cultured for 24 h, and the conditioned medium was subjected to a sandwich enzyme-linked immunosorbent assay using three types of specific monoclonal antibodies as described previously (35, 39).

We monitored the activity of γ- and β-secretase as reported previously (40, 41). Solubilized membranes were incubated overnight at 37 °C in 200 μl of 50 mM Tris-HCl (pH 6.8) buffer containing 2 mM EDTA, 0.25% CHAPSO (w/v), and 10 μM fluorescent substrate of γ-secretase or were incubated for 1 h at 37 °C in 200 μl of 50 mM acetate buffer (pH 4.1) containing 100 mM sodium chloride, 0.025% bovine serum albumin, and 10 μM fluorescent β-secretase substrate. We measured fluorescence using a plate reader (Fluostar Galaxy) with an excitation wavelength of 355 nm and an emission wavelength of 440 nm (for the γ-secretase) or 510 nm (for the β-secretase).

Small Interfering RNA (siRNA) Targeting of Genes—We used siRNA with the sequence 5′-gcuggaaaacucuucagadTdT-3′ and 5′-ucugaagaguuuucccagcdTdT-3′, 5′-gcaagcaaguccuaacauudTdT-3′ and 5′-aauguuaggacuugcuugcdTdT-3′, or 5′-cgguuccagucucucggugdAdG-3′ and 5′-caccgagagacuggaaccgdAdT-3′ as annealed oligonucleotides for repressing clathrin, Rab5, or Rab7 expression, respectively. Cells were transfected with siRNA using HiPerFect transfection reagent according to the manufacturer's instructions. Non-silencing siRNA (5′-uucuccgaacgugucacgudTdT-3′ and 5′-acgugacacguucggagaadTdT-3′) was used as a negative control.

Immunostaining Microscopy—Cells or mouse brain sections were incubated with antibody against each protein for 30 min before (for HA or EP4) or after (for PS-1 and Rab7) treatment with PGE_2. Samples were fixed and incubated with the respective secondary antibody. We acquired images with a confocal fluorescence microscope (Olympus FV500).

Co-immunoprecipitation Assay—Immunoprecipitation was carried out as described previously (35), with some modifications. Cells were harvested, lysed with buffer containing 1% CHAPSO, and centrifuged. The antibody against HA or EP_4 was added to the supernatant, and the samples were incubated for 12 h at 4 °C with rotation. Dynabeads-Protein G was added and incubated for 2 h at 4 °C with rotation. Beads were washed four times, and the proteins were eluted by boiling in SDS sample buffer.

Surface Biotinylation Assay—This assay was carried out as described previously (42) with some modifications. Proteins on the cell surface were biotinylated with a reversible membrane-impermeable derivative of biotin (sulfo-NHS-S-S-biotin). Internalization of proteins was allowed to occur by incubation at 37 °C for 1 h. The remaining cell surface biotin was cleaved by glutathione, and the cells were lysed. Biotinylated proteins were precipitated using UltraLink immobilized Neutravidin beads and eluted by boiling in SDS sample buffer.

Statistical Analysis—All values are expressed as the mean ± S.D. Two-way analysis of variance followed by the Tukey test or the Student's *t* test for unpaired results was used to evaluate differences between more than three groups or between two

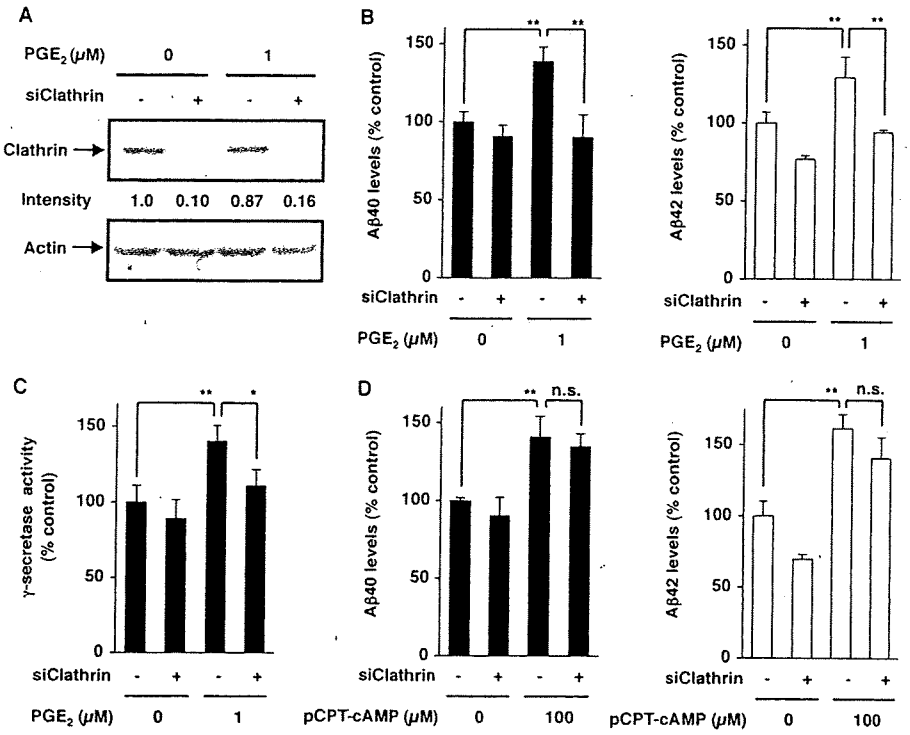

FIGURE 2. **Effect of siRNA for clathrin on PGE_2-stimulated production of Aβ.** HEK293 cells expressing APPsw were transiently transfected with siRNA for clathrin (*siClathrin*) (+) or non-silencing siRNA (−) (*A–D*). Cells were incubated with 1 μM PGE_2 (*A–C*) or 100 μM pCPT-cAMP (*D*) for 24 h (*A*, *B*, and *D*) or 1 h (*C*). Whole cell extracts were analyzed by immunoblotting with an antibody against clathrin or actin. The band intensity was determined and expressed relative to the control (*A*). The amounts of Aβ (*B* and *D*) and the γ-secretase activity (*C*) were determined and expressed as described in the legend for Fig. 1. Values are given as means ± S.D. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant.

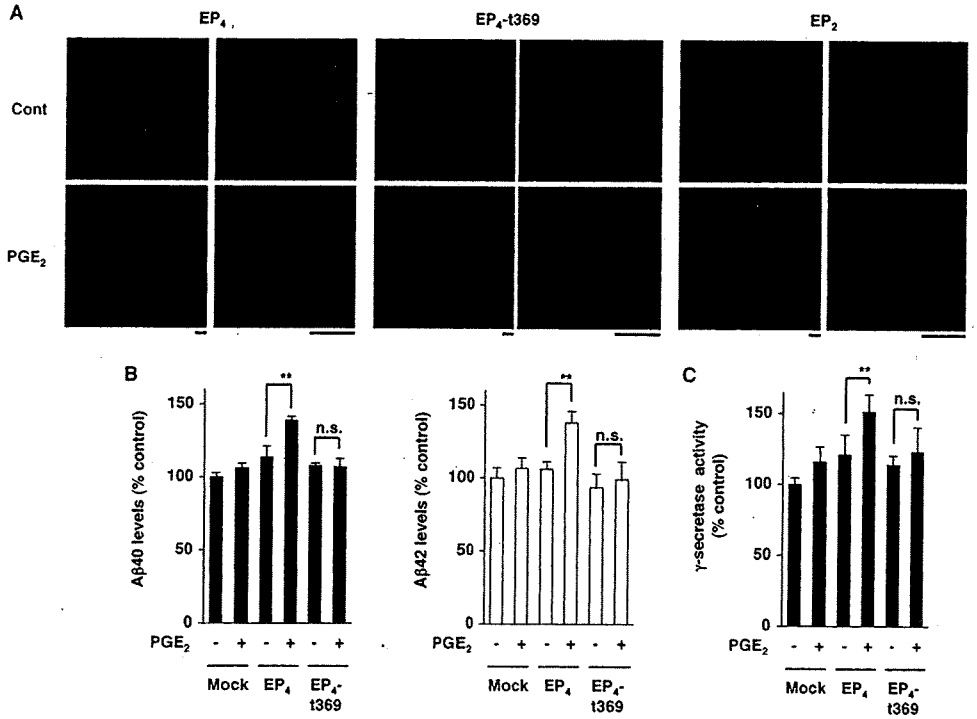

FIGURE 3. **PGE_2-dependent internalization of the EP_4 receptor and its contribution to PGE_2-stimulated production of Aβ.** CHO-K1 cells expressing APPsw were transiently transfected with expression plasmid encoding EP_2 (*A*), EP_4 (*A–C*), or EP_4-t369 (*A–C*) or with control vector (*B* and *C*). Cells were preincubated for 1 h with antibody against HA and further cultured for 1 h (*A* and *C*) or 24 h (*B*) with or without 1 μM PGE_2. After incubation with secondary antibody, the cells were inspected using fluorescence microscopy. Pictures of both high (*right*) and low (*left*) magnification are shown (*scale bar*, 20 μm) in each of the three panels. (*A*) The amounts of Aβ (B) or γ-secretase activity (C) were determined and expressed as described in the legend for Fig. 1. Values are given as means ± S.D. ($n = 3$). **, $p < 0.01$; *n.s.*, not significant.

groups, respectively. Differences were considered to be significant for values of $p < 0.05$.

RESULTS

Mechanism for EP_2 Receptor-mediated Stimulation of Aβ Production by PGE_2—Although primary neurons should be used for this type of experiments, we used an immortalized cell line in this study, because we used stable and transient transfection for the experiments in this study (see below). We confirmed our previous results that H-89 did not block PGE_2-stimulated production of Aβ in HEK293 cells and found that an inhibitor of adenylate cyclase, SQ22536, also did not block this stimulation (supplemental Fig. S1, *A* and *B*), suggesting that an increase in the cellular level of cAMP is not involved in PGE_2-stimulated production of Aβ in HEK293 cells. In contrast, in SH-SY5Y cells, both H-89 and SQ22536 decreased the level of Aβ in the presence, but not in the absence, of PGE_2 (supplemental Fig. S1, *C* and *D*). These inhibitors at the concentrations used did not affect cell viability (data not shown). Therefore, the results shown in Fig. 1, *C* and *D*, suggest that an increase in the cellular level of cAMP and the resulting activation of PKA are involved in PGE_2-stimulated production of Aβ in SH-SY5Y cells. In a previous report (30), we used HEK293 cells that express only APPsw stably. In the current study, we used CHO and HEK293 cells that express each EP receptor transiently in addition to the stable expression of APPsw (see below). For these types of cells, 10 nM PGE_2, which had been used for experiments in the previous study (30), was not enough to stimulate the production of Aβ clearly (supplemental Figs. S7–S10), and thus we used 1 μM PGE_2 in all experiments described in this article.

As the EP_2 receptor is involved in PGE_2-stimulated production of Aβ in SH-SY5Y but not in HEK293 cells (30), the results shown in supplemental Fig. S1 suggest that the

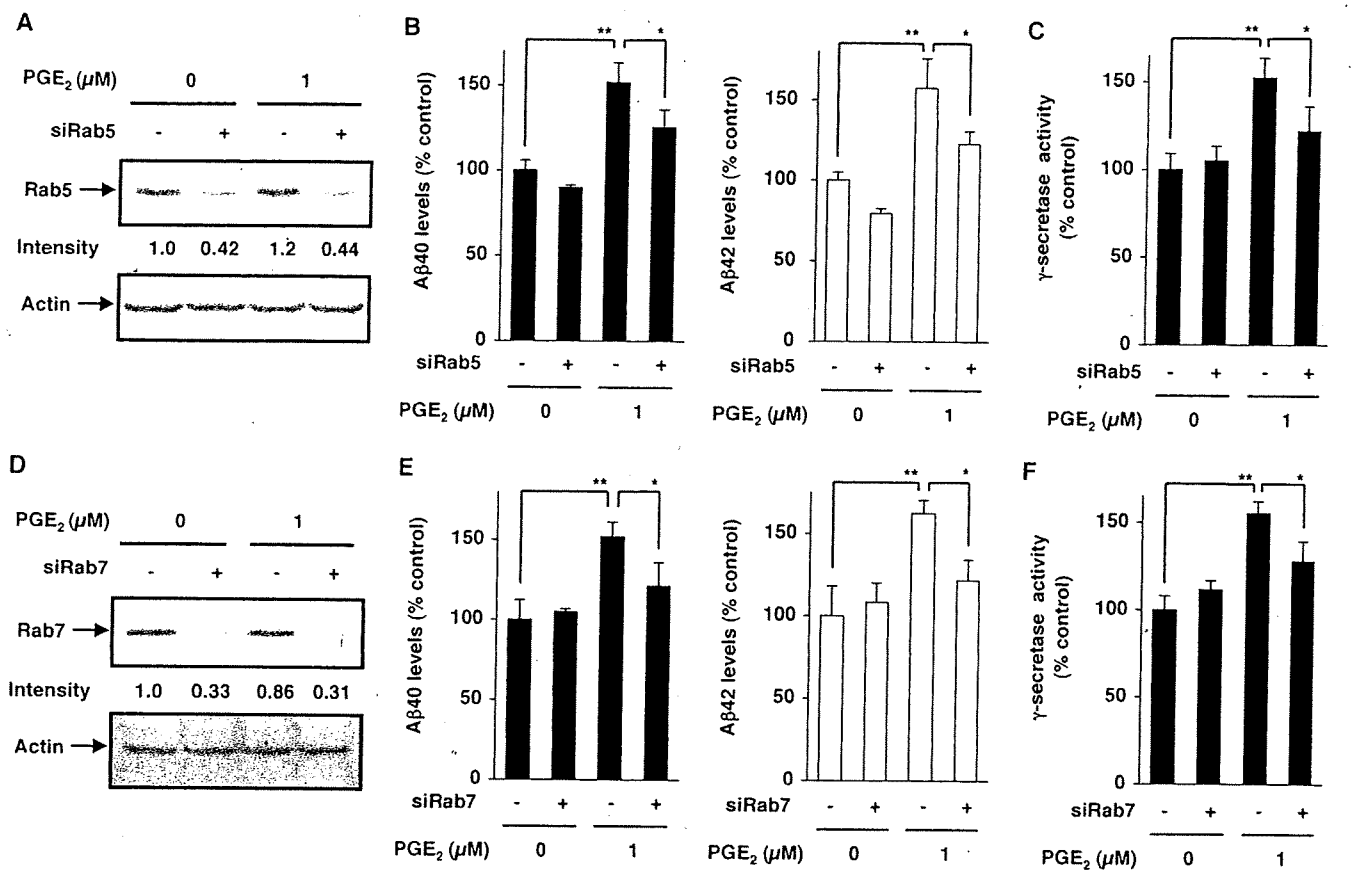

FIGURE 4. **Effect of siRNA for Rab5 and Rab7 on PGE_2-stimulated production of Aβ.** HEK293 cells expressing APPsw were transiently transfected with siRNA for Rab5 (siRab5) (*A–C*) or Rab7 (siRab7) (*D–F*), or with non-silencing siRNA (−) (*A–F*). Cells were incubated with 1 μM PGE_2 for 24 h (*A, B, D,* and *E*) or 1 h (*C* and *F*). Whole cell extracts were analyzed by immunoblotting as described in the legend for Fig. 2 (*A* and *D*). The amounts of Aβ (*B* and *E*) and γ-secretase activity (*C* and *F*) were determined and expressed as described in the legend for Fig. 1. Values are given as means ± S.D. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$.

increase in the cellular level of cAMP and resulting activation of PKA contributes to EP_2 receptor-dependent (but not EP_4 receptor-dependent) stimulation of Aβ production by PGE_2. To confirm this proposition, we examined the effects of these inhibitors on the production of Aβ in CHO cells artificially expressing APPsw and EP_2 or EP_4 receptor tagged with HA. Lack of functional endogenous EP_2 and EP_4 receptors in cells has been reported previously (43). Expression of these receptors was confirmed by immunoblotting (data not shown) and immunostaining (Fig. 3*A*). Both H-89 and SQ22536 suppressed PGE_2-stimulated production of Aβ in cells expressing the EP_2 receptor but not in cells expressing the EP_4 receptor (supplemental Fig. S2, *A–D*), supporting our theory described above. We reported previously that treatment of HEK293 cells with PGE_2 increases γ-secretase activity in extracts of these cells (30). Here we found that PGE_2 treatment increased γ-secretase activity in extracts prepared from CHO cells expressing the EP_4 receptor but not in cells expressing the EP_2 receptor and that it did not affect β-secretase activity in either of these cell types (supplemental Fig. S2, *E* and *F*). Furthermore, pCPT-cAMP did not affect γ-secretase activity in HEK293 cells (data not shown). On the other hand, treatment of cells with PGE_2 did not affect the expression of γ-secretase (PS-1-NTF) (supplemental Fig. S3). These results suggest that the EP_2 receptor mediates PGE_2-stimulated production of Aβ through activation of cAMP and PKA without an increase in β- and γ-secretase activity and that the EP_4 receptor mediates PGE_2-stimulated Aβ production through different mechanisms, which involve an activation rather than induction of expression of γ-secretase.

Mechanism for EP_4 Receptor-mediated Stimulation of Aβ Production by PGE_2—Agonist-dependent internalization (endocytosis) of the EP_4 receptor (but not the EP_2 receptor) has been described: The binding of PGE_2 to the EP_4 receptor induces formation of vesicles that contain the receptor and the vesicles are trafficked to endosomes (44, 45). Thus, we used inhibitors of endocytosis (sucrose and concanavalin A) to test whether agonist-dependent internalization of the EP_4 receptor is involved in PGE_2-stimulated production of Aβ. As shown in Fig. 1, both sucrose and concanavalin A suppressed PGE_2 (1 μM)-stimulated production of Aβ and decreased γ-secretase activity in HEK293 cells. Similar results were obtained with 10 nM PGE_2 in HEK293 cells (supplemental Fig. S7) and in CHO cells expressing the EP_4 receptor but not in those expressing the EP_2 receptor (data not shown). These inhibitors, at the concentrations specified in Fig. 1, did not affect cell viability (data not shown). These results suggest that agonist-dependent internalization of the EP_4 receptor is involved in PGE_2-stimulated Aβ production.

Agonist-dependent internalization is initiated by the formation of clathrin-coated vesicles, and thus clathrin is essential for this internalization (46). We examined the effect of siRNA targeting the clathrin heavy chain on PGE_2-stimulated production

of Aβ in HEK293 cells. Transfection with siRNA inhibited clathrin expression in the presence and absence of PGE_2 (Fig. 2*A*). This siRNA suppressed PGE_2-stimulated production of Aβ and γ-secretase activity (Fig. 2, *B* and *C*), suggesting that clathrin-dependent vesicle formation at the cell surface and their subsequent internalization are involved in EP_4 receptor-mediated stimulation of Aβ production by PGE_2. siRNA did not affect Aβ production in the absence of PGE_2 (Fig. 2*B*) and its pCPT-cAMP-dependent stimulation (Fig. 2*D*), suggesting that siRNA specifically affects EP_4 receptor-mediated stimulation of Aβ production by PGE_2.

By immunostaining we observed PGE_2 (1 μM)-dependent internalization of EP_4 receptors (Fig. 3*A*). In contrast, EP_2 receptors remained localized to the cell surface even after PGE_2 treatment (Fig. 3*A*). It has been reported that the C-terminal region of the EP_4 receptor is required for its agonist-dependent internalization (45). We confirmed that a mutant form of the EP_4 receptor, which is truncated after Thr-369 (EP_4-t369) (47), does not exhibit agonist-dependent internalization (Fig. 3*A*). As shown in Fig. 3, *B* and *C*, in contrast to CHO cells expressing the wild-type EP_4 receptor, in CHO cells expressing EP_4-t369, PGE_2 did not stimulate the production of Aβ and γ-secretase activity. Similar results were obtained with 10 nM PGE_2; however, the effects were not so apparent, and some of them were not statistically significant (supplemental Fig. S8). This suggests that agonist-dependent internalization of the EP_4 receptor is essential for EP_4 receptor-mediated and PGE_2-dependent stimulation of Aβ production and γ-secretase activation.

In clathrin-dependent endocytosis, vesicles formed at the cell surface are trafficked first to early endosomes and then to LEL. Rab5 and Rab7 are essential for the traffic to early endosomes and LEL, respectively (48). To examine the role of the traffic in PGE_2-stimulated Aβ production, the effects of siRNA for Rab5 and Rab7 on the production of Aβ were examined. Each siRNA clearly inhibited the expression of their target protein (Fig. 4, *A* and *D*). Furthermore, siRNA for Rab5 or Rab7 suppressed the PGE_2-stimulated production of Aβ and γ-secretase activity in HEK293 cells (Fig. 4, *B*, *C*, *E*, and *F*), suggesting that traffic of vesicles containing the EP_4 receptor to LEL is important for PGE_2-stimulated Aβ production and γ-secretase activity.

Contribution of Co-internalization of PS-1 with the EP_4 Receptor to PGE_2-stimulated Production of Aβ—It was reported recently that γ-secretase is activated when it is trafficked into endosomes via agonist-dependent internalization of the β-adrenergic receptor, which interacts with γ-secretase (49). Thus, we hypothesized that the EP_4 receptor also interacts with γ-secretase and that γ-secretase is trafficked to LEL in a PGE_2-dependent manner, resulting in activation of γ-secretase and stimulation of Aβ production. To test this theory, we first examined the interaction between the EP_4 receptor and PS-1 by a co-immunoprecipitation assay. As shown in Fig. 5*A*, efficient immunoprecipitation of PS-1-NTF with antibody against was dependent on the expression of the HA-tagged EP_4 receptor. On the other hand, PS-1-NTF was not immunoprecipitated with antibody against HA in cells expressing HA-tagged EP_2 receptor (supplemental Fig. S4). These results suggest that the EP_4 receptor can physically and specifically interact with PS-1-NTF (γ-secretase). The physical interaction between EP_4 receptor and PS-1-NTF was also observed in SH-SY5Y cells without artificial overexpression of EP_4 receptor (supplemental Fig. S11*A*). PS-1 is cleaved to produce PS-1-NTF and PS-1-CTF in cells, and both of PS-1-NTF and PS-1-CTF are included in γ-secretase complex. The results in Fig. 5 also show that EP_4-t369 can interact with PS-1-NTF, suggesting that the interaction of PS-1 (γ-secretase) with the EP_4 receptor alone is not sufficient for PGE_2-stimulated production of Aβ and γ-secretase activity (see Fig. 3, *B* and *C*). It has been reported that a general acceleration of cellular endocytic pathways and agonist-induced endocytosis of some receptors (such as the angiotensin II receptor) does not affect the production of Aβ and γ-secretase activity (49, 50). Thus, internalization of PS-1 (γ-secretase) with the EP_4 receptor seems to enhance production of Aβ and γ-secretase activity specifically.

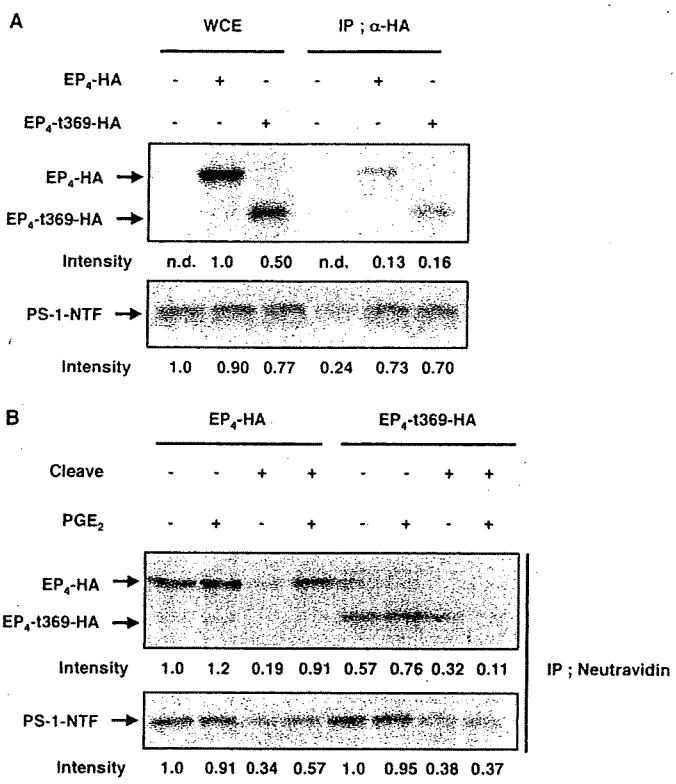

FIGURE 5. **Interaction between the EP_4 receptor and PS-1 and their PGE_2-dependent co-internalization.** HEK293 cells expressing APPsw were transiently transfected with expression plasmid encoding the EP_4 receptor or EP_4-t369 or with control vector. Whole cell extracts were immunoprecipitated with antibody against HA. *A*, whole cell extracts (*WCE*) and the immunoprecipitates (*IP*) were analyzed by immunoblotting with antibody against HA or PS-1-NTF as described in the legend for Fig. 2 (*n.d.*, not detectable). *B*, cells were surface-biotinylated and incubated with or without 1 μM PGE_2. Then, cells were treated with glutathione to cleave biotin from the surface proteins. Biotinylated proteins present in the cell lysates were precipitated with Neutravidin, and the precipitates were analyzed by immunoblotting with HA or PS-1-NTF as described in the legend for Fig. 2.

Next, we tested co-internalization of PS-1-NTF with the EP_4 receptor using a surface biotinylation assay. Cells were surface-biotinylated, and after induction of internalization, biotinylated proteins remaining on the cell surface were cleaved by glutathione. The biotinylated proteins (internalized proteins) were precipitated, and the presence of each protein in the precipitates was monitored by immunoblotting. As shown in Fig. 5*B*, the

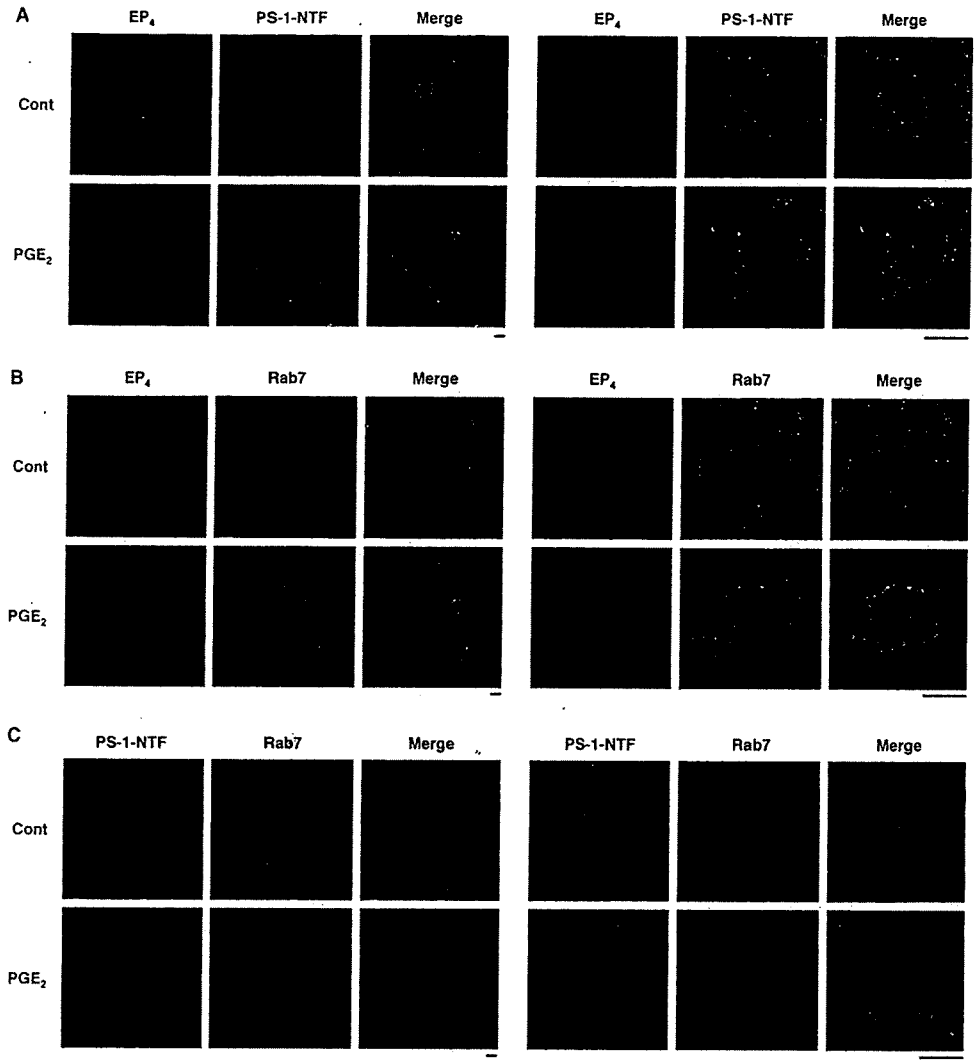

FIGURE 6. **Co-localization of EP_4 receptors, PS-1, and Rab7 in cells.** HEK293 cells expressing APPsw were transiently transfected with an expression plasmid encoding the EP_4 receptor. Cells were preincubated with antibody against HA (*A*, *B*) for 1 h and further incubated for 1 h with or without (*Cont*, control) 1 μM PGE_2. After fixation, samples were incubated with antibody against PS-1-NTF (*A*, *C*) or Rab7 (*B*, *C*). After incubation with the respective secondary antibody, cells were inspected using fluorescence microscopy as described in the legend for Fig. 3.

wild-type EP_4 receptor and EP_4-t369 bands were not apparent after glutathione cleavage, whereas preincubation of cells with PGE_2 (1 μM) prior to cleavage gave rise to the wild-type EP_4 receptor band but not the EP_4-t369 band. This shows that the wild-type EP_4 receptor, but not EP_4-t369, internalizes in a PGE_2-dependent manner. The PS-1-NTF band was also not apparent following cleavage; preincubation with PGE_2 recovered the band in cells expressing wild-type EP_4 receptor but not in cells expressing EP_4-t369 (Fig. 5*B*). Similar results were obtained with 10 nM PGE_2 in HEK293 cells (supplemental Fig. S9) and in SH-SY5Y cells without artificial overexpression of EP_4 receptor (supplemental Fig. S11*B*). We also examined the effect of sucrose and concanavalin A on a surface biotinylation assay for EP_4 receptor and PS-1-NTF. As shown in supplemental Fig. S5, preincubation of cells with PGE_2 prior to cleavage did not restore the band of either the EP_4 receptor or PS-1-NTF in the presence of sucrose or concanavalin A, confirming that these inhibitors suppressed the endocytosis. Similar results were observed for transfection of siRNA for clathrin or Rab5 but not for Rab7 (supplemental Fig. S6). These results suggest that internalization of PS-1 as a result of PGE_2 treatment is dependent on internalization of the EP_4 receptor.

For further confirmation of PGE_2-dependent co-internalization of the EP_4 receptor and PS-1, we performed a co-immunostaining assay. Similar to what was observed for the EP_4 receptor, PS-1-NTF was localized to the cell surface in the absence of PGE_2; however, strong staining of PS-1-NTF in intracellular components was observed after PGE_2 treatment (Fig. 6*A*). As shown in Fig. 6*A* (see *merged panel*), localizations of the EP_4 receptor and PS-1-NTF were well matched, suggesting that PS-1-NTF co-internalizes with the EP_4 receptor.

To identify the intracellular components where both PS-1-NTF and the EP_4 receptor localize after PGE_2 treatment, we performed a co-immunostaining assay for these factors with Rab7, an LEL marker. As shown in Fig. 6, *B* and *C*, intracellular localizations of the EP_4 receptor (and PS-1-NTF) and Rab7 were well matched in PGE_2-treated cells but not in control cells, suggesting that PS-1 is trafficked into LEL with the EP_4 receptor in a PGE_2 treatment-dependent manner. Results similar to those in Fig. 6 (in HEK293 cells with 1 μM PGE_2) were obtained with 10 nM PGE_2 in HEK293 cells (supplemental Fig. S10) and in SH-SY5Y cells without artificial overexpression of EP_4 receptor (supplemental Fig. S12).

Finally, we tested the *in vivo* relevance of our *in vitro* results using transgenic mice expressing APPsw (APP23) that were crossed to $EP_4^{-/-}$ mice (APPsw/$EP_4^{-/-}$ mice). We had previously reported that the amount of Aβ in the brains of APPsw/$EP_4^{-/-}$ mice was lower than in APPsw/$EP_4^{+/+}$ mice (30). As shown in Fig. 7*A*, the γ-secretase activity in extracts prepared from the brains of APPsw/$EP_4^{-/-}$ mice was very slightly, but significantly, lower than in those from APPsw/$EP_4^{+/+}$ mice. This is consistent with the *in vitro* results, which showed that PGE_2 increases γ-secretase activity in extracts in an EP_4 receptor-dependent manner (supplemental Fig. 2*F*). There was no significant difference of the expression of PS-1-NTF between APPsw/$EP_4^{-/-}$ and APPsw/$EP_4^{+/+}$ mice (Fig. 7*B*), suggesting that activation rather than expression of γ-secretase is important for the decrease in the amount of Aβ in the brains of APPsw/$EP_4^{-/-}$. We previously reported that in HEK293 cells where the EP_4 but not the EP_2 receptor is functional, PGE_2 does

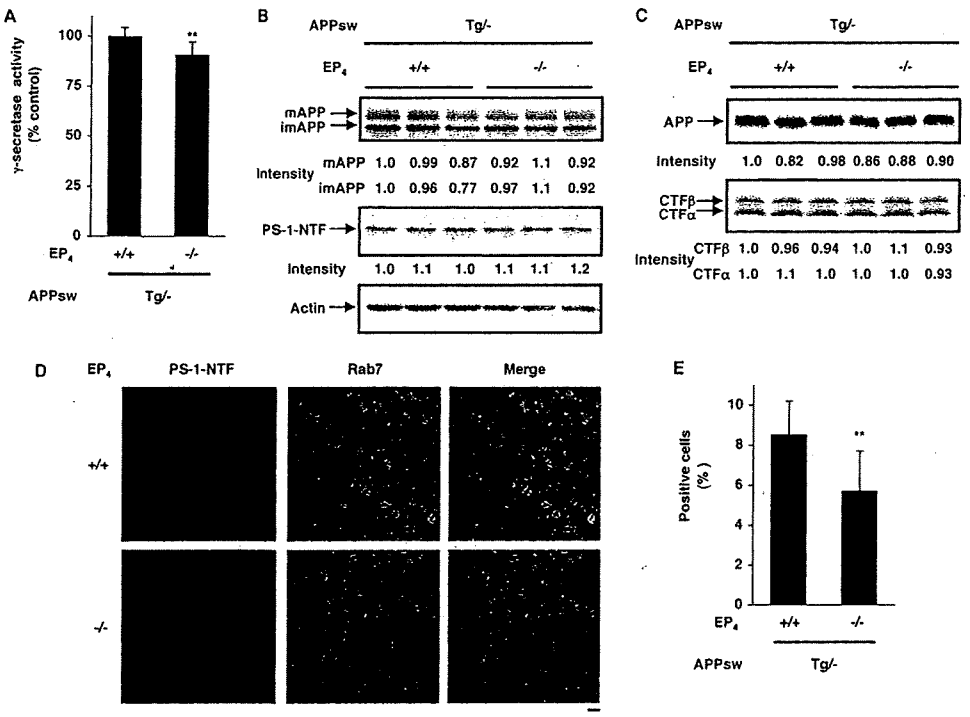

FIGURE 7. **Mechanism for EP_4 receptor-mediated stimulation of production of Aβ *in vivo*.** Membrane fractions (*A* and *C*) or whole cell extracts (*B*) were prepared from the brains of 3-month-old APPsw/$EP_4^{+/+}$ and APPsw/$EP_4^{-/-}$ mice and subjected to a γ-secretase-mediated peptide cleavage assay (*A*) or immunoblotting with antibody against APP, PS-1-NTF or actin (*B* and *C*). Values are given as means ± S.D. ($n = 6$). **, $p < 0.01$ (*A*). The brain sections were prepared from the same mice and subjected to immunostaining as described in the legend for Fig. 6 (*D*). The ratio of cells with *yellow spots* (positive cells) to total cells in the brain sections (three sections/brain) was determined. Values are given as means ± S.D. ($n = 8$). **, $p < 0.01$ (*E*).

not affect the expression and maturation of APP or α- and β-secretase activities (30). In this study, we showed that the expression and maturation of APP (the ratio of the mature form of APP (mAPP) to the immature form of APP (imAPP)) and the α- and β-secretase activities in extracts prepared from the brains of APPsw/$EP_4^{-/-}$ mice were similar to those from APPsw/$EP_4^{+/+}$ mice (Fig. 7*B* and *C*). (CTFs of APP that are generated by α- or β-secretase (CTFα or CTFβ, respectively) are used as an indirect index of secretase activity.) Furthermore, we compared the co-localization of PS-1-NTF with Rab7 in brain sections prepared from these mice. As shown in Fig. 7, *D* and *E*, *yellows spots* (index of the intracellular co-localization of PS-1-NTF with Rab7) were more apparent in brain sections prepared from APPsw/$EP_4^{+/+}$ mice than in sections from APPsw/$EP_4^{-/-}$ mice, suggesting that even *in vivo* PS-1 is trafficked to LEL in an EP_4 receptor-dependent manner. The results shown in Fig. 7 suggest that our *in vitro* results are relevant *in vivo* and that PGE_2-dependent traffic of PS-1 into LEL contributes to the observed increase in γ-secretase activity and resulting stimulation of Aβ production by expression of the EP_4 receptor *in vivo*.

DISCUSSION

We recently reported the importance of PGE_2 as a factor that connects inflammation and AD; PGE_2 stimulates production of Aβ and this stimulation is mediated by EP_2 and EP_4 receptors. We also suggested the importance of EP_2 and EP_4 receptors in production of Aβ *in vivo* by showing that the amount of Aβ in the brains of APPsw/$EP_2^{-/-}$ and APPsw/$EP_4^{-/-}$ mice was lower than in the respective control mice. Based on these results, we proposed that antagonists for EP_2 and/or EP_4 receptor would be therapeutically beneficial for AD (30). To determine the potential drug target (whether it is the EP_2 receptor, the EP_4 receptor, or both), it is important to understand the molecular mechanism for intracellular signal transduction governing EP_2 (or EP_4) receptor-mediated stimulation of Aβ production by PGE_2. Because activation of both EP_2 and EP_4 receptors is coupled to activation of the cAMP-PKA pathway, we speculated that this pathway might be involved in the signal transduction. However, our attempts to prove this conjecture failed in our previous study (30). Furthermore, our finding that both EP_2 knock-out mice and EP_4 receptor knock-out mice showed decreased levels of Aβ in the brain could not be explained by the hypothesis that the cAMP-PKA pathway is responsible for both EP_2 receptor- and EP_4 receptor-mediated signal transduction pathways for PGE_2 stimulated Aβ production. In the current study, using inhibitors for adenylate cyclase and PKA, we showed that the cAMP-PKA pathway is involved in EP_2-mediated but not EP_4-mediated stimulation of Aβ production by PGE_2. As the EP_4 receptor is not linked to the cAMP-PKA pathway for PGE_2-stimulated Aβ production, EP_4 receptor antagonists may be therapeutically beneficial for the treatment of AD. It has been reported that the cAMP-PKA pathway is important for long-term potentiation (51, 52) so EP_2 receptor, but not EP_4 receptor, antagonists may have side effects on the memory system by inhibiting long-term potentiation. On the other hand, deletion of EP_2 receptor in mice was shown to decrease oxidative damage and inhibit β-secretase, resulting in a decrease in the level of Aβ in brain (29), or to enhance Aβ phagocytosis in the brain (53). It has also been suggested that PGE_2-dependent activation of cAMP-PKA pathway causes induction of expression of APP (54, 55), indicating that the EP_2 receptor antagonists may be therapeutically beneficial for the treatment of AD. The fact that EP_2 and EP_4 receptors stimulate the production of Aβ through different mechanisms could explain why single knock-outs of either the EP_2 or EP_4 receptor reduce Aβ production *in vivo*.

Much attention has been paid to agonist-dependent internalization (endocytosis) of receptors, including the EP_4 receptor, because this causes receptor desensitization. Furthermore, this internalization was also recently reported to be important for signal transduction (44). From the current study, we propose that the EP_4 receptor mediates the PGE_2 signal through its co-internalization with PS-1 (γ-secretase). This suggestion is based on the following results: The immunoprecipitation assay

revealed a physical interaction between the EP_4 receptor and PS-1-NTF; PGE_2-stimulated production of Aβ was not observed under conditions in which co-internalization of the EP_4 receptor and PS-1 is inhibited (such as in the presence of endocytosis inhibitors, in cells transfected with siRNA for clathrin, and in cells expressing EP_4-t369); internalization of PS-1-NTF was observed to be dependent on both PGE_2 and expression of the wild-type EP_4 receptor in the surface biotinylation assay. This is the first demonstration of the EP_4 receptor mediating signal transduction through co-internalization with other molecules. Similar mechanisms may be involved in EP_4 receptor-mediated signal transduction for other responses.

We have also concluded that PS-1 (γ-secretase) is trafficked to LEL in a PGE_2- and EP_4 receptor-dependent manner and that this traffic is important for PGE_2-stimulated production of Aβ and γ-secretase activity. This conclusion is based on the following results: PGE_2-dependent co-localization of PS-1-NTF, EP_4, and Rab7 was observed *in vitro*, and co-localization of PS-1-NTFand Rab7 was observed in APPsw/$EP_4^{+/+}$ mice but not as distinctly in APPsw/$EP_4^{-/-}$ mice; and transfection of siRNA for Rab5 or Rab7 inhibited PGE_2-stimulated production of Aβ and γ-secretase activity. A similar mechanism was proposed for β-adrenergic receptor-mediated stimulation of production of Aβ and γ-secretase activity (49). They also showed the enhancement of γ-secretase activity and elevation of Aβ production in endosomes. It is well known that the pH in endosomes is relatively low and that as a result γ-secretase is more active in endosomes (56–58). Thus, the relatively low pH value in endosomes may contribute to EP_4 receptor-mediated stimulation of Aβ production. However, we found that the γ-secretase activity in extracts decreased when the extracts were prepared from cells cultured under conditions in which the traffic of PS-1 (γ-secretase) into endosomes is inhibited, even though the γ-secretase assay was carried out at the same pH. Thus, something other than the lower pH of endosomes (such as induction of expression and post-translational modification of γ-secretase) may also contribute to the stimulation of Aβ production by internalization of PS-1 (γ-secretase) into endosomes. It is also possible that internalization of the EP_4 receptor stimulates the traffic of β-secretase to endosomes, resulting in its activation, as is the case for apolipoprotein receptor-2 (59), because β-secretase is also more active at lower pH (60). However, we reported previously that the activity of β-secretase (estimated from the amounts of CTFβ) is not enhanced by PGE_2 (30). Thus, the EP_4 receptor may not interact with β-secretase.

In summary, we have shown that EP_2 and EP_4 receptors mediate stimulation of production of Aβ by PGE_2 through distinct mechanisms. This finding is important for understanding the mechanisms underlying the inflammation-mediated progression of AD and the regulation of vesicle transport of PS-1 (γ-secretase) and for identifying potential targets for the development of drugs to treat AD.

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