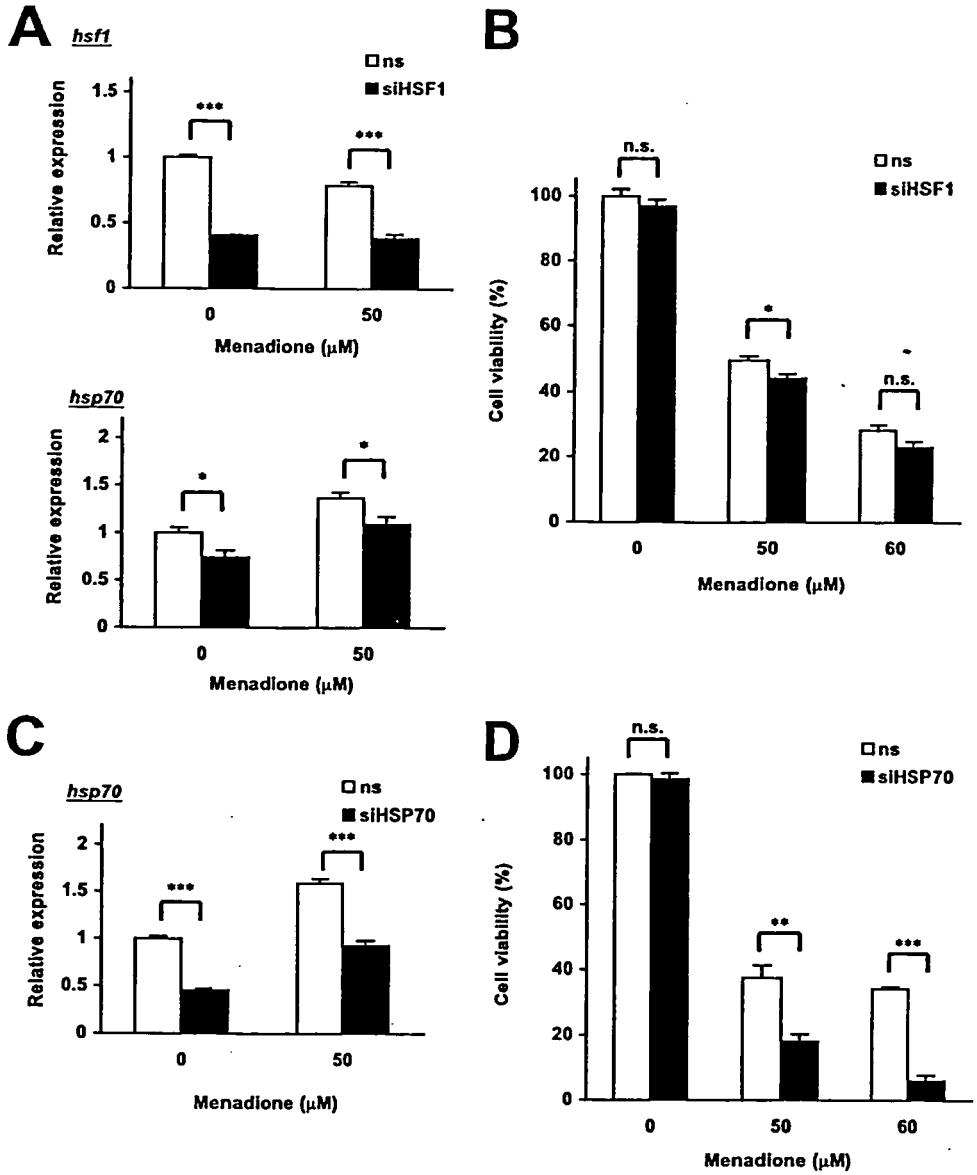

FIGURE 9. **Effect of siRNAs for HSF1 and HSP70 on menadione-induced cell death.** HCT-15 cells were transfected with 1.2 μg of siRNA for HSF1 (*siHSF1*; *A* and *B*), siRNA for HSP70 (*siHSP70*; *C* and *D*), or non-silencing siRNA (*ns*; *A–D*). After 24 h, cells were incubated with the indicated concentrations of menadione for 1 h. The relative expression of each gene was monitored as described in the legend of Fig. 2. Values were normalized to the actin gene, expressed relative to the control sample, and are given as the mean ± S.E. ($n = 3$) (*A* and *C*). Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (*B* and *D*). Values shown are the mean ± S.E. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *n.s.*, not significant.

in vivo. On the other hand, HSF1 siRNA did not stimulate the LPS-induced mRNA expression of *madcam-1*, and HSP70 siRNA did not stimulate the LPS-induced mRNA expression of all of these CAMs. These observations suggest that the alterations to the mRNA expression of these CAMs seen *in vivo* are achieved indirectly, for example, through up-regulation of TNF-α, which has been reported to induce the expression of these CAMs both *in vivo* and *in vitro* (10).

Colonic mucosal cell death induced by ROS released from activated leukocytes is thought to be directly responsible for the pathogenesis of human IBD and DSS-induced colitis (2). Analysis using the TUNEL assay revealed that cell death in colonic mucosa was stimulated or inhibited in HSF1-null mice or transgenic mice expressing HSP70, respectively. This correlates with other parameters for DSS-induced colitis; however, it was not clear whether these alterations to cell death cause or result from the progression of DSS-induced colitis. Given that transfection with siRNA for HSP70 stimulated ROS-induced cell death *in vitro*, this result suggests that HSP70 protects colonic mucosal cells from ROS-induced cell death, which seems to contribute to the lower level of cell death seen in the colonic mucosa of DSS-administered transgenic mice expressing HSP70. On the other hand, transfection with siRNA specific for HSF1 did not stimulate ROS-induced cell death *in vitro* to the same extent. Thus, the higher level of cell death seen in the colonic mucosa of DSS-administered HSF1-null mice is the result (rather than the cause) of aggravation of DSS-induced colitis.

The results of this study suggest that nontoxic inducers of HSP expression are therapeutically beneficial for IBD. Supporting this notion, geranylgeranylacetone (a leading anti-ulcer drug in the Japanese market and a nontoxic HSP inducer) (53) suppresses both DSS- and trinitrobenzenesulfonic acid-induced colitis (54, 55). However, the ability of geranylgeranylacetone to induce HSP expression is not strong, which may explain its relatively weak effect on these types of colitis (54, 55). Therefore, we propose that more potent nontoxic HSP inducers would be therapeutically beneficial for IBD.

Acknowledgments—We thank Drs. C. E. Angelidis and G. N. Pagoulatos for generously providing the mice.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 45, pp. 32670–32688, November 9, 2007

Involvement of Prostaglandin E_2 in Production of Amyloid-β Peptides Both *in Vitro* and *in Vivo*[*] [S]

Received for publication, April 12, 2007, and in revised form, August 29, 2007 Published, JBC Papers in Press, September 2, 2007, DOI 10.1074/jbc.M703087200

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Amyloid-β peptides (Aβ), generated by proteolysis of the β-amyloid precursor protein (APP) by β- and γ-secretases, play an important role in the pathogenesis of Alzheimer disease (AD). Inflammation is also believed to be integral to the pathogenesis of AD. Here we show that prostaglandin E_2 (PGE_2), a strong inducer of inflammation, stimulates the production of Aβ in cultured human embryonic kidney (HEK) 293 or human neuroblastoma (SH-SY5Y) cells, both of which express a mutant type of APP. We have demonstrated using subtype-specific agonists that, of the four main subtypes of PGE_2 receptors (EP_{1-4}), EP_4 receptors alone or EP_2 and EP_4 receptors together are responsible for this PGE_2-stimulated production of Aβ in HEK293 or SH-SY5Y cells, respectively. An EP_4 receptor antagonist suppressed the PGE_2-stimulated production of Aβ in HEK293 cells. This stimulation was accompanied by an increase in cellular cAMP levels, and an analogue of cAMP stimulated the production of Aβ, demonstrating that increases in the cellular level of cAMP are responsible for the PGE_2-stimulated production of Aβ. Immunoblotting experiments and direct measurement of γ-secretase activity suggested that PGE_2-stimulated production of Aβ is mediated by activation of γ-secretase but not of β-secretase. Transgenic mice expressing the mutant type of APP showed lower levels of Aβ in the brain, when they were crossed with mice lacking either EP_2 or EP_4 receptors, suggesting that PGE_2-mediated activation of EP_2 and EP_4 receptors is involved in the production of Aβ *in vivo* and in the pathogenesis of AD.

Alzheimer disease (AD)[2] is the leading cause of adult onset dementia, with a dramatic increase in the incidence of AD apparent in our rapidly aging society. AD is characterized pathologically by the accumulation of tangles and senile plaques. Senile plaques are composed of the amyloid-β peptides (Aβ) Aβ40 and Aβ42 (1, 2). Aβ is generated by secretase-dependent proteolysis of the β-amyloid precursor protein (APP). Prior to proteolysis, APP undergoes modifications, such as glycosylation and phosphorylation. To generate Aβ40 and Aβ42, APP is first cleaved by β-secretase and then by γ-secretase. For the cleavage of APP, β-secretase competes with α-secretase, which 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). The early onset familial AD is linked to three genes, *APP*, *PS1*, and *PS2* (5, 6), strongly suggesting that the production of Aβ, which reflects the proteolysis of APP by secretases (particularly γ-secretase), is a key factor in the pathogenesis of AD. Therefore, cellular factors that stimulate the production of Aβ may be good drug targets for the prevention and treatment of AD.

It has been repeatedly suggested that inflammation is important in the pathogenesis of AD. Chronic inflammation, which is indicated by accumulation of microglia around senile plaques and elevated levels of inflammatory cytokines, chemokines, proteases, and reactive oxygen species, has been observed in the brains of AD patients (7). Furthermore, trauma to the brain and ischemia, both of which can activate inflammation, are major risk factors for AD (8).

Prostaglandins (PGs), one of the major groups of chemical mediators in the mammalian body, are potent inducers of inflammation (9). Cyclooxygenase (COX) is essential for the synthesis of PGs and has two subtypes, COX-1 and COX-2. COX-1 is expressed constitutively in most cell types, whereas expression of COX-2 is induced by various factors including inflammatory cytokines and is responsible for the progression

* This work was supported by grants-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of Japan, as well as the Japan Science and Technology Agency, and grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5 and additional references.

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[2] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid-β peptides; APP, β-amyloid precursor protein; PS, presenilin; CHO, Chinese hamster ovary; COX, cyclooxygenase; CTF, C-terminal fragment; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester; EIA, enzyme immunoassay; Epac, exchange protein directly activated by cAMP; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide; HEK, human embryonic kidney; NF-κB, nuclear factor-κB; NSAIDs, non-steroidal anti-inflammatory drugs; pCPT-cAMP, 8-(4-chlorophenylthio)-cAMP; pCPT-*O*-Me-cAMP, 8-(4-chloro-phenylthio)-2′-*O*-methyladenosine-3′-5′-cyclic monophosphate; PI3K, phosphatidylinositol 3-kinase; PGE_2, prostaglandin E_2; PGs, prostaglandins; Rock, Rho kinase; PKA, protein kinase A; RT, reverse transcriptase; sELISA, sandwich enzyme-linked immunosorbent; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

of inflammation (10, 11). Elevated levels of PGE_2, a major proinflammatory product of COX, and overexpression of COX-2 have been observed in the brains of AD patients (12–14). It has also been reported that the extent of COX-2 expression correlates with the amount of Aβ and the degree of progression of AD pathogenesis (15). Furthermore, transgenic mice that constitutively overexpress COX-2 have been reported to show stimulation of aging-dependent neural apoptosis and memory dysfunction (16). These previous studies suggest that COX-2 and PGE_2 are important in the pathogenesis of AD and are therefore good targets for potential AD drugs. Supporting this notion, epidemiological studies have revealed that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs), inhibitors of COX, delays the onset and reduces the risk of AD (17). In an animal model of AD, administration of some NSAIDs decreased the amount of Aβ and senile plaques and suppressed microglial activation (18–20). Furthermore, in cultured cells, treatment with NSAIDs decreased the amount of Aβ (21, 22). Therefore, NSAIDs have attracted much attention as a new class of drugs for the treatment of AD. However, clinical use of NSAIDs is associated with various side effects, such as gastrointestinal complications (23) and cardiovascular thrombotic events (24, 25). These side effects are mainly due to an NSAID-induced nonspecific decrease in the levels of various types of prostanoids and eicosanoids and the inhibition of signal transduction mediated by their receptors. Identification of specific prostanoids and eicosanoids, or of their receptors that are involved in the anti-AD activity of NSAIDs, is therefore important for the development of new types of drugs for AD with a reduced risk of side effects.

Based on the studies described above, it is reasonable to hypothesize that PGE_2 increases the amount of Aβ. In fact, it was recently reported that PGE_2 stimulates the production of Aβ in Chinese hamster ovary (CHO) cells (26). However, the molecular mechanism governing this stimulation has remained unclear. For example, whereas PGE_2 receptors have been pharmacologically subdivided into four main subtypes (EP_1, EP_2, EP_3, and EP_4) (27), the EP subtype involved in this PGE_2-stimulated production of Aβ has not been identified. In the present study, we have confirmed that PGE_2 stimulates the production of Aβ in human embryonic kidney (HEK) 293 and human neuroblastoma (SH-SY5Y) cells. Experiments with EP agonists and antagonists have revealed that, depending on cell type, the EP_4 receptor alone or the EP_2 and EP_4 receptors together are involved in the PGE_2-stimulated production of Aβ. Furthermore, experiments with transgenic mice suggest that EP_2 and EP_4 receptors are also involved in the production of Aβ *in vivo*. Based on the results of the current study, we propose that antagonists for both EP_2 and EP_4 receptors may be therapeutically beneficial for the treatment of AD.

EXPERIMENTAL PROCEDURES

Materials—Compounds used in this study are listed in Table 1. Dulbecco's modified Eagle's medium and Ham's F-12 medium were obtained from Nissui Pharmaceutical Co. The EIA (enzyme immunoassay) kit for cAMP measurement and the first-strand cDNA synthesis kit were from GE Healthcare. Lipofectamine (TM2000) and the pcDNA3.1 plasmid were purchased from Invitrogen. HilyMax was from Dojindo Laboratories. The plasmids pcDNA3.1/APPsw, pcDNA3/APP695 and pcDNA3/APP695 T668A were from our laboratory stocks (28, 29). The plasmid pEGFP-N1 was obtained from Clontech. Antibodies against actin or Thr-668 phosphorylated APP were obtained from Santa Cruz or Cell Signaling, respectively. An antibody against EP_2 receptor was from Cayman Chemical. Fetal bovine serum, PGE_2, LY294002, pCPT-cAMP, pCPT-O-Me-cAMP, G418, 3-isobutyl-1-methylxanthine, H-89 and an antibody against the C-terminal fragment of APP were from Sigma. DI-004, AE1-259, AE-248, AE1-329, 8713, AE3-240, and AE3-208 were from our laboratory stocks. The RNeasy kit was 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) and DAPT were from the Peptide Institute Inc. *Taq* DNA Polymerase was from TAKARA.

Animals—APP23 transgenic mice were a gift from Dr. M. Staufenbiel; these mice were generated as previously described (30).

APP23 mice were crossed with $EP_2^{-/-}$ mice (31) to generate APPsw/$EP_2^{+/-}$ mice and these mice were again crossed to $EP_2^{-/-}$ mice to generate APPsw/$EP_2^{-/-}$ mice. Parallel crosses were made between APPsw mice and C57BL/6 mice (wild type mice for $EP_2^{-/-}$ mice) to generate APPsw/$EP_2^{+/+}$ control mice.

Most $EP_4^{-/-}$ mice die in the C57BL/6 background. Therefore, survivors of the F_2 progenies of $EP_4^{-/-}$ mice in the mixed genetic background of 129/Ola and C57BL/6 were intercrossed and the resulting female survivors were used as described (32, 33). APP23 mice were crossed to these $EP4^{-/-}$ mice to generate APPsw/$EP_4^{+/-}$ mice and these were crossed to $EP4^{-/-}$ mice to generate APPsw/$EP_4^{-/-}$ mice. Parallel crosses were made between APPsw mice and mice in the mixed genetic background of 129/Ola and C57BL/6 to generate APPsw/$EP_4^{+/+}$ control mice. 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 or SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium or Dulbecco's modified Eagle's medium/Ham's F-12 medium, respectively, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. HEK293 and SH-SY5Y cells expressing APPsw were from our laboratory stocks (34).

For transient expression of each gene, cells were seeded 24 h before the transfection in 24-well plates at a density of 1.5×10^5 cells/well. The transfection was carried out using Lipofectamine (TM2000) or HilyMax 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 expressing each gene were selected by immunoblotting or real-time RT-PCR analyses.

Positive clones were maintained in the presence of 200 μg/ml G418.

Immunoblotting Analysis—Whole cell extracts were prepared as described previously (35). For detection of the C-terminal fragment (CTF) α and CTFβ, membrane fractions were prepared as described previously (36). For detection of CTFγ, the membrane fractions were incubated for 2 h at 37 °C. The protein concentration of each sample was determined by the Bradford method (37). Samples were applied to polyacrylamide SDS gels (Tris-Tricine gel for the detection of APP and Tris glycine gel for other proteins), and subjected to electrophoresis, after which proteins were immunoblotted with their respective antibodies.

Sandwich Enzyme-linked Immunosorbent Assay (sELISA) for Aβ and EIA for cAMP—Cells were cultured for 24 h and the conditioned medium was subjected to sELISA using three types of specific monoclonal antibodies, as described previously (29, 34).

The amount of Aβ in mouse brain was determined as described previously (38). Briefly, the brain hemispheres were homogenized in 50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl with a homogenizer (Polytron) and centrifuged at 200,000 × *g* for 20 min at 4 °C. The supernatant, defined as the soluble fraction, was taken and guanidine-HCl added to give a final concentration of 0.5 M before sELISA. The pellet was solubilized by sonication in 6 M guanidine-HCl buffer. The solubilized pellet was centrifuged at 200,000 × *g* for 20 min at 4 °C, and the resulting supernatant was diluted and termed the insoluble fraction. The amounts of Aβ40 and Aβ42 in each fraction were determined by sELISA.

Cells were pre-treated for 30 min with 0.5 M 3-isobutyl-1-methylxanthine (an inhibitor of phosphodiesterase) and further cultured for 10 min with or without PGE_2, EP agonists, or EP antagonist. Cells were lysed with ice-cold 100% ethanol and centrifuged. The supernatants were dried, re-suspended in the assay buffer, and applied to the EIA kit for measurement of cAMP, according to the manufacturer's instructions.

RT-PCR Analysis—Total RNA was extracted from cells using an RNeasy kit according to the manufacturer's protocols. Samples (10 μg of RNA) were reverse transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was amplified by PCR (TAKARA PCR Thermal Cycler) using TAKARA *Taq* DNA polymerase, and reaction products were analyzed by agarose gel electrophoresis. PCR cycle conditions were 2 min at 50 °C, followed by 10 min at 95 °C and finally 35 cycles at each of 95 °C for 20 s, 60 °C for 60 s, and 72 °C for 60 s.

Primers were designed using the Primer3 Web site (www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of the primers (written as target cDNA: forward primer and reverse primer) were: *EP1*, 5′-accttctttggcggctct-3′ and 5′-gcacgacaccaccatgatac-3′; *EP2*, 5′-ccacctcattctcctg-gcta-3′ and 5′-cgacaacagaggactgaacg-3′; *EP3*, 5′-agcttatggggat-catgtgc-3′ and 5′-tctgcttctccgtgtgtgtc-3′; *EP4*, 5′-tgcgagtat-tcgtcaaccag-3′ and 5′-ggtctaggatggggttcaca-3′; and *actin*, 5′-ggacttcgagcaagagatgg-3′ and 5′-agcactgtgttggcgtacag-3′.

γ-Secretase-mediated Peptide Cleavage Assay—We performed the assay as previously reported (39, 40). Solubilized membranes were re-suspended and incubated overnight at 37 °C in 200 μl of assay buffer containing 50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 0.25% CHAPSO (w/v), and 10 μM APP-derived fluorescent substrate of γ-secretase. We measured fluorescence using a plate reader (Fluorstar Galaxy) with an excitation wavelength of 355 nm and an emission wavelength of 440 nm.

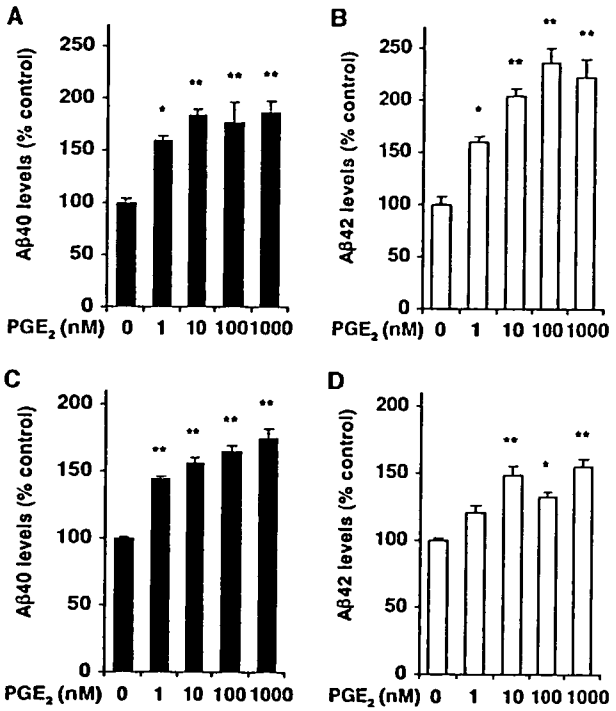

FIGURE 1. **Stimulation of production of Aβ by PGE_2.** HEK293 (*A* and *B*) or SH-SY5Y (*C* and *D*) cells expressing APPsw were cultured for 24 h with the indicated concentrations of PGE_2. The amounts of Aβ40 and Aβ42 in the conditioned medium were determined by sELISA and expressed relative to the control (without PGE_2). Values are given as mean ± S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. Similar results in this figure were obtained in two other independent experiments.

Statistical Analysis—All values are expressed as the mean ± S.E. One-way analysis of variance followed by Tukey multiple comparison test or Student's *t* test for unpaired results was used for evaluation of differences among more than three groups or for the evaluation of differences between two groups, respectively. Differences were considered to be significant for values of $p < 0.05$.

RESULTS

Stimulation of Aβ Production by PGE_2—We used HEK293 cells that stably express a form of APP with double mutations (K651N/M652L), known as the "Swedish" mutations (APPsw) (29). These mutations elevate cellular and secreted levels of Aβ (29). The amount of these peptides in conditioned medium was determined using a sELISA. Treatment of cells with PGE_2 increased the levels of Aβ (Aβ40 and Aβ42) in the conditioned medium (Fig. 1, *A* and *B*), a similar result to that observed for CHO cells (26). We concluded that this increase is due to stimulation of production of Aβ because, after treatment of the cells with PGE_2, not only did the amount of secreted Aβ increase but the amount of Aβ in the cells also increased and because pulse label experiments with [^{35}S]methionine showed that treatment

of cells with PGE_2 increased generation of CTFs of APP that are co-generated by γ-secretase (CTFγ) with Aβ (data not shown). Although it is not clear whether comparison of concentrations of PGE_2 between *in vivo* and *in vitro* is reasonable or not, the concentrations of PGE_2 required for stimulation of Aβ production (1–10 nM) are within the same range of PGE_2 concentrations that are observed in the human brain (41). However, the fact that production of Aβ is stimulated by the concentration of PGE_2, which is equivalent to that found physiologically is against the idea that PGE_2-stimulated production of Aβ seen *in vitro* is involved in inflammation-stimulated development of AD. We also performed similar experiments in SH-SY5Y cells that stably express APPsw. As shown in Fig. 1, *C* and *D*, treatment with PGE_2 also increased the level of Aβ in SH-SY5Y cells; however, the extent of stimulation of Aβ production in SH-SY5Y cells was not as dramatic as that seen in HEK293 cells (Fig. 1, *A* and *B*). We confirmed that PGE_2 did not affect the cell growth and intracellular lactate dehydrogenase activity at concentrations used in Fig. 1 in both HEK293 and SH-SY5Y cells (supplemental Fig. S1).

TABLE 1
Compounds used in this study

Compound	Primary action
DI-004	EP_1 agonist
AE1–259	EP_2 agonist
AE-248	EP_3 agonist
AE1–329	EP_4 agonist
8713	EP_1 antagonist
AE3–240	EP_3 antagonist
AE3–208	EP_4 antagonist
pCPT-cAMP	cAMP analogue
LY294002	PI3K inhibitor
H-89	PKA inhibitor
pCPT-*O*-Me-cAMP	Epac activator
DAPT	γ-Secretase inhibitor

Identification of EP Receptors Involved in PGE_2-stimulated Production of Aβ—We used agonists specific for each EP receptor (Table 1) to identify EP receptors involved in the PGE_2-stimulated production of Aβ. Initially, we used HEK293 cells and examined the mRNA expression of each EP receptor by RT-PCR. As shown in Fig. 2*A*, mRNA for each of the EP receptors was detected, although the level of expression varied between them. We also confirmed the expression of EP_2 receptor by immunoblotting experiments (Fig. 5*A*). Then, we examined the effect of agonists specific for each EP receptor on the production of Aβ in HEK293 cells. Treatment of cells with AE1-329 (an EP_4 agonist) increased the level of Aβ in conditioned medium (Fig. 2, *H* and *I*). The amplitude of this increase

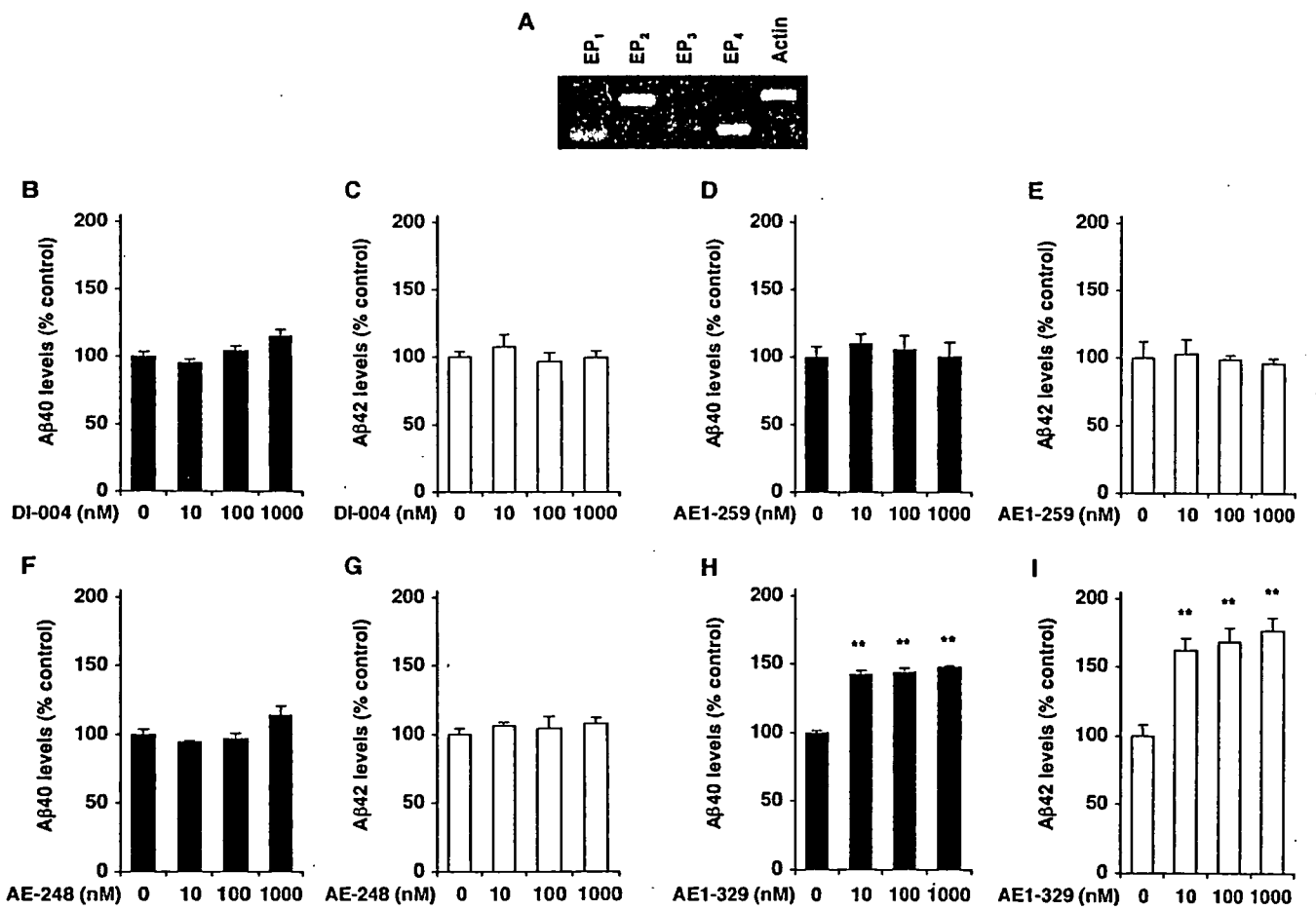

FIGURE 2. **Effect of EP receptor agonists on production of Aβ in HEK293 cells.** To determine mRNA expression of each EP receptor, total RNA was extracted from HEK293 cells expressing APPsw and subjected to RT-PCR by use of a specific primer set for each gene. Actin was used as a control. Reaction products were analyzed by agarose (2%) gel electrophoresis (*A*). HEK293 cells expressing APPsw were cultured for 24 h with the indicated concentrations of DI-004 (EP_1 agonist) (*B* and *C*), AE1-259 (EP_2 agonist) (*D* and *E*), AE-248 (EP_3 agonist) (*F* and *G*), or AE1-329 (EP_4 agonist) (*H* and *I*). The amounts of Aβ40 and Aβ42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean ± S.E. ($n = 3$). **, $p < 0.01$. Similar results in this figure were obtained in two other independent experiments.

was similar to that achieved with PGE_2 (Fig. 1, *A* and *B*). Based on previously reported findings using AE1-329, it is reasonable to postulate that, for the concentrations used in the experiment described in Fig. 2, *H* and *I*, it acts as a specific agonist for the EP_4 receptor (42). In contrast, none of the other specific agonists, including DI-004 (an EP_1 agonist), AE1-259 (an EP_2 agonist), and AE-248 (an EP_3 agonist), significantly affected the level of Aβ (Fig. 2, *B–G*). Based on previous reports, the concentrations of EP agonists employed in the experiments described in Fig. 2 should have been sufficient to activate their respective EP receptor (42). We confirmed that each agonist did not affect the cell growth at these concentrations used in Fig. 2 (data not shown). Consequently, the results in Fig. 2 suggest that EP_4 is responsible for the PGE_2-stimulated production of Aβ in HEK293 cells. As described below, we suggested that EP_2 receptor is not functional in HEK293 cells (Fig. 5*A*). Furthermore, we found that none of PGE_2, DI-004, and AE-248 increased the intracellular Ca^{2+} levels (supplemental Fig. S2), suggesting that neither EP_1 nor EP_3 receptor is functional in HEK293 cells (both EP_1 and EP_3 receptors are coupled to Ca^{2+} mobilization (27, 43)). Thus, we could not conclude that activation of these receptors (EP_1, EP_2, and EP_3) does not affect the level of Aβ in general, based on the inability of DI-004, AE1-259, and AE-248 to affect the level of Aβ in HEK293 cells.

To further confirm this conclusion, we examined the effect of antagonists specific for each EP receptor (Table 1) on the PGE_2-stimulated production of Aβ. As shown in Fig. 3, *E* and *F*, AE3-208 (an EP_4 antagonist) clearly suppressed the PGE_2-dependent increase in the level of Aβ in a dose-dependent manner. Based on previous reports, it is reasonable to postulate that, for the concentrations used in the experiment described in Fig. 3, *E* and *F*, AE3-208 acts as a specific antagonist for the EP_4 receptor (32). This EP_4 antagonist (1 or 10 μM) decreased the level of Aβ to below background (Fig. 3, *E* and *F*). This seems to be due to inhibition of the action of endogenous PGE_2, because this antagonist decreased the level of Aβ even in the absence of exogenously added PGE_2 (data not shown). In contrast, 8713 (an EP_1 antagonist), at concentrations previously reported to antagonize the EP_1 receptor (44), did not significantly affect the level of Aβ in the presence of PGE_2 (Fig. 3, *A* and *B*). AE3-240 (an EP_3 antagonist) weakly inhibited the PGE_2-stimulated production of Aβ42 only at the highest concentration tested (10 μM) (Fig. 3, *C* and *D*). This seems to be due to the tendency of AE3-240 to cross-react with the EP_4 receptor at high concentrations, as has been described previously (45). We confirmed that each antagonist did not affect the cell growth at these concentrations used in Fig. 3 (data not shown). Therefore, the results obtained from the experiments with EP antagonists support the idea that EP_4 is responsible for the PGE_2-stimulated production of Aβ in HEK293 cells.

We also examined the effect of each EP agonist on production of Aβ in SH-SY5Y cells. Again, mRNA for each of the EP receptors was detected by RT-PCR (Fig. 4*A*). However, the relative expression of each EP receptor was different from that in HEK293 cells (Fig. 2*A*). For example, the amount of EP_4 receptor mRNA relative to EP_2 receptor mRNA was less in SH-SY5Y cells than in HEK293 cells (Figs. 2*A* and 4*A*). We also confirmed the expression of EP_2 receptor by immunoblotting experiments

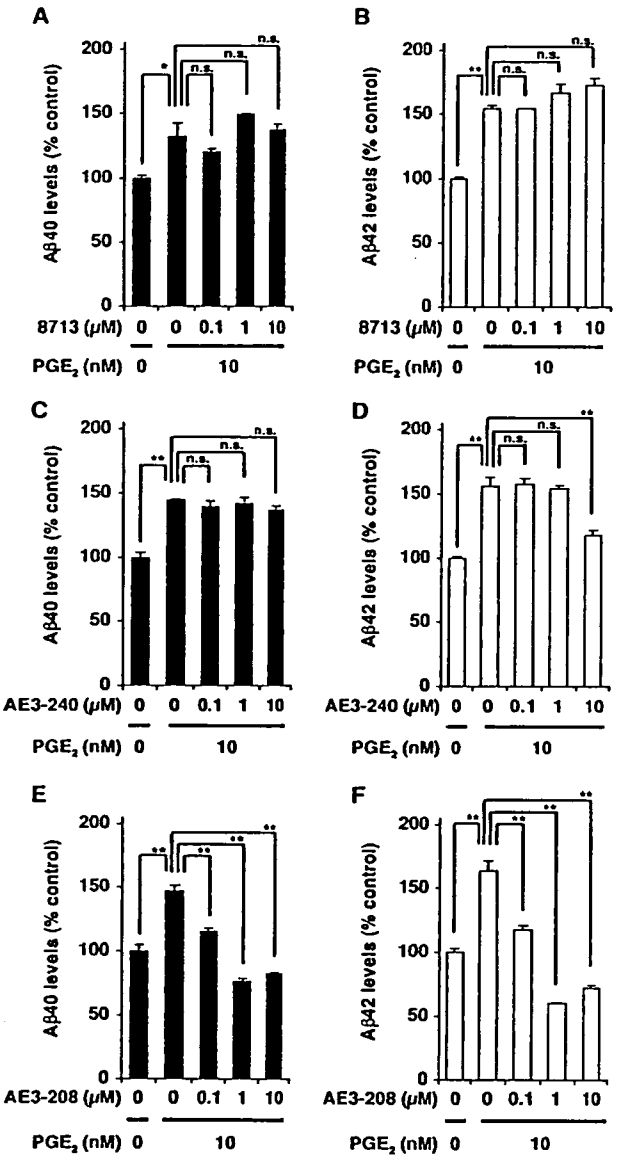

FIGURE 3. **Effect of EP receptor antagonists on PGE_2-stimulated production of Aβ in HEK293 cells.** HEK293 cells expressing APPsw were cultured for 24 h with the indicated concentrations of 8713 (EP_1 antagonist) (*A* and *B*), AE3-240 (EP_3 antagonist) (*C* and *D*), or AE3-208 (EP_4 antagonist) (*E* and *F*) in the presence of PGE_2 (10 nM). The amounts of Aβ40 and Aβ42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean ± S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant. Similar results in this figure were obtained in two other independent experiments.

(Fig. 5*A*). As shown in Fig. 4, *B* and *C*, not only the EP_4 agonist but also the EP_2 agonist increased the level of Aβ in the conditioned medium of SH-SY5Y cells. Again, EP_1 or EP_3 agonists did not significantly affect the level of Aβ (Fig. 4, *B* and *C*). We confirmed that each agonist did not affect the cell growth at these concentrations used in Fig. 4 (data not shown). These results suggest that both EP_2 and EP_4 receptors are involved in PGE_2-stimulated production of Aβ in SH-SY5Y cells, and therefore differs from that observed for HEK293 cells. As shown in supplemental Fig. S2, each of all PGE_2, DI-004, and AE-248 increased the intracellular Ca^{2+} levels in SH-SY5Y cells, suggesting that both EP_1 and EP_3 receptors are functional

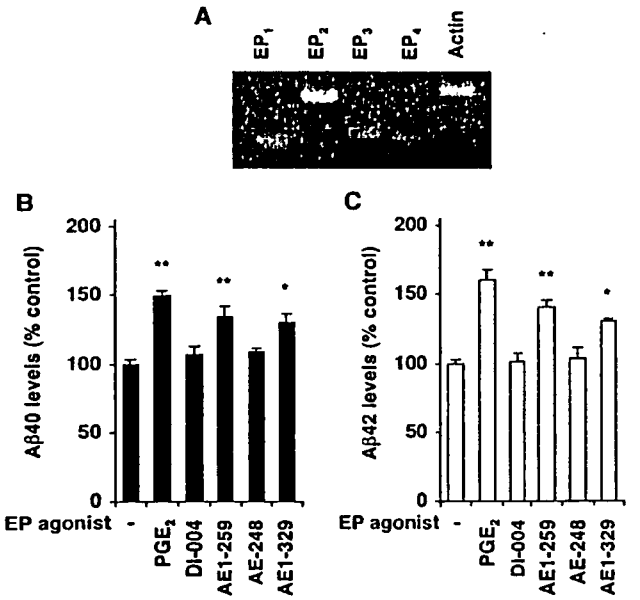

FIGURE 4. **Effect of EP receptor agonists on production of Aβ in SH-SY5Y cells.** The mRNA expression of each EP receptor in SH-SY5Y cells expressing APPsw was examined as described in the legend of Fig. 2 (*A*). SH-SY5Y cells expressing APPsw were cultured for 24 h with 1 μM DI-004 (EP_1 agonist), AE1-259 (EP_2 agonist), AE-248 (EP_3 agonist), AE1-329 (EP_4 agonist), or PGE_2. The amounts of Aβ40 and Aβ42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean ± S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. *B* and *C*, similar results in this figure were obtained in two other independent experiments.

in the cells. Thus, results in Fig. 4 suggest that activation of EP_1 and EP_3 receptors does not affect the level of Aβ.

Involvement of the Cellular Level of cAMP in PGE_2-stimulated Production of Aβ—Activation of EP_2 and EP_4 receptors causes activation of adenylate cyclase activity and an increase in the cellular level of cAMP (27). Therefore, the results described above suggest that an increase in the cellular level of cAMP is involved in PGE_2-stimulated production of Aβ. To test this hypothesis, we monitored the cellular level of cAMP under conditions where production of Aβ is stimulated or suppressed (Figs. 1–4). In HEK293 cells PGE_2 and EP_4 agonist, but not EP_2 agonist, increased the cellular level of cAMP (Fig. 5*B*). However, PGE_2, EP_2 agonist, and EP_4 agonist all increased the cAMP level in SH-SY5Y cells (Fig. 5*C*), correlating with the stimulation of Aβ production shown in Figs. 2 and 4. These data suggest that both EP_2 and EP_4 receptors are functional in SH-SY5Y, whereas only EP_4 receptor is functional in HEK293 cells, which can explain why both EP_2 and EP_4 agonists or only EP_4 agonist stimulated production of Aβ in SH-SY5Y or HEK293, respectively (Figs. 2 and 4). As shown in Fig. 5*A*, the expression of EP_2 receptor was relatively higher in SH-SY5Y than HEK293 cells. It is also possible that this low expression of EP_2 receptor in HEK293 cells can explain why this type of cells were inert for the increase in the cellular level of cAMP by EP_2 agonist. Furthermore, EP_4 antagonist clearly suppressed the PGE_2-mediated increase in the cellular level of cAMP, again correlating with the trend in Aβ production shown in Fig. 3. Therefore, the results in Fig. 5, *B–D*, suggest that PGE_2-stimulated production of Aβ is mediated by an increase in the cellular level of cAMP.

To further confirm this conclusion a cAMP analogue, 8-(4-chlorophenylthio)-cAMP (pCPT-cAMP), was used. As shown

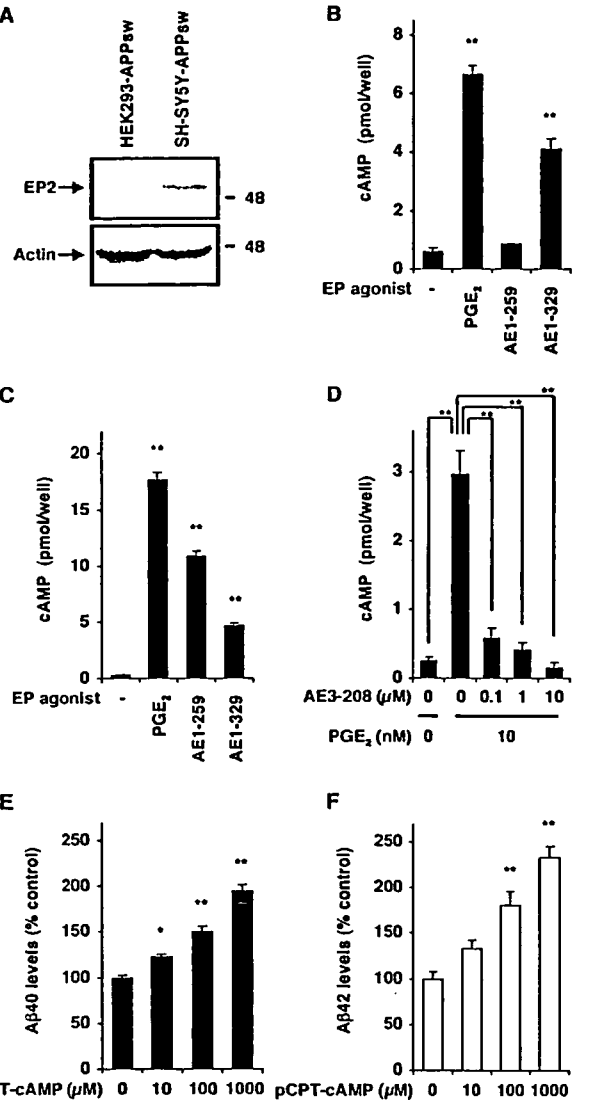

FIGURE 5. **Involvement of the cellular level of cAMP in PGE_2-stimulated production of Aβ.** To determine the expression of EP_2 receptor, cell extracts were prepared from HEK293 or SH-SY5Y cells expressing APPsw and subjected to immunoblotting with an antibody against EP_2 receptor or actin (*A*). HEK293 (*B* and *D*) or SH-SY5Y (*C*) cells expressing APPsw were pre-treated for 30 min with 0.5 M 3-isobutyl-1-methylxanthine and further cultured for 10 min with 1 μM PGE_2, AE1-259 (EP_2 agonist), AE1-329 (EP_4 agonist) (*B* and *C*), or with the indicated concentrations of AE3-208 (EP_4 antagonist) in the presence of PGE_2 (10 nM) (*D*). Cellular cAMP levels were determined by EIA (*B–D*). HEK293 cells expressing APPsw were cultured for 24 h with the indicated concentrations of pCPT-cAMP (*E* and *F*). The amounts of Aβ40 and Aβ42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean ± S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. Similar results in this figure were obtained in two two independent experiments.

in Fig. 5, *E* and *F*, pCPT-cAMP increased the level of Aβ in HEK293 cells and the extent of this increase was similar to that achieved with PGE_2 (Fig. 1, *A* and *B*), supporting the idea that an increase in the cellular level of cAMP is responsible for PGE_2-stimulated production of Aβ. We confirmed that pCPT-cAMP did not affect cell growth at these concentrations as used in Fig. 5 (data not shown).

An increase in the cellular level of cAMP is known to activate two types of kinases, protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI3K), which are important for cAMP-regu-

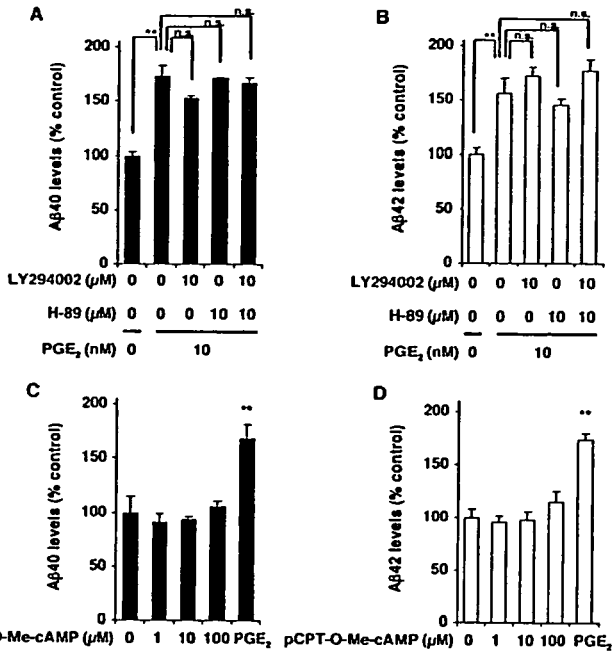

FIGURE 6. **Effect of inhibitors or activators of various cAMP-regulated molecules on PGE_2-stimulated production of Aβ.** HEK293 cells expressing APPsw were preincubated for 1 h with the indicated concentrations of LY294002 (PI3K inhibitor) and/or H-89 (PKA inhibitor) and further cultured for 24 h with 10 nM PGE_2 in the presence of the same concentration of each inhibitor as in the preincubation step (*A* and *B*). Cells were cultured for 24 h with the indicated concentrations of pCPT-*O*-Me-cAMP (Epac activator) (*C* and *D*). The amounts of Aβ40 and Aβ42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean ± S.E. ($n = 3$). **, $p < 0.01$; *n.s.*, not significant. Similar results in this figure were obtained in two other independent experiments.

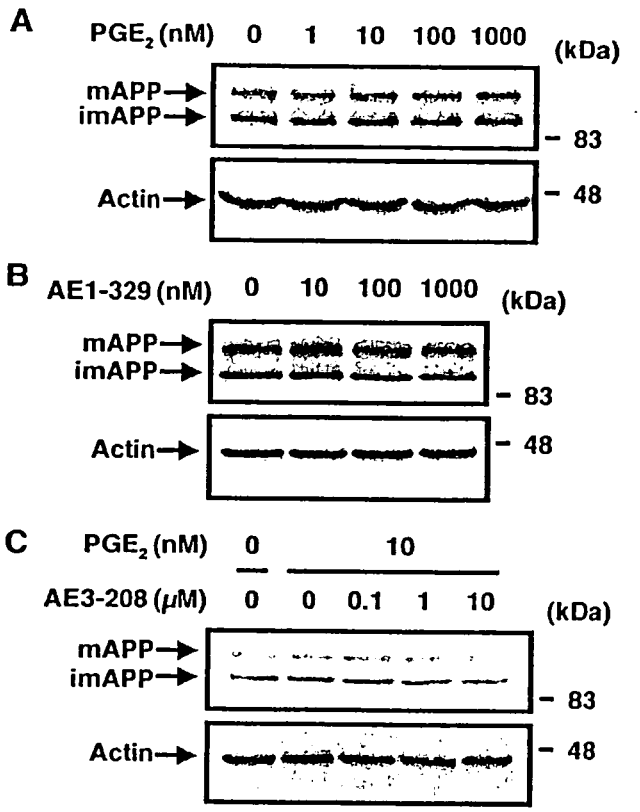

FIGURE 7. **Effect of PGE_2, EP_4 agonist, and EP_4 antagonist on the maturation of APP.** HEK293 cells expressing APPsw were cultured for 24 h in the presence of the indicated concentrations of PGE_2 alone (*A*), AE1-329 (EP_4 agonist) (*B*), or PGE_2 plus AE3-208 (EP_4 antagonist) (*C*). Whole cell extracts (10 μg of protein) were analyzed by immunoblotting with an antibody against the C-terminal fragment of APP or actin. Similar results in this figure were obtained in two other independent experiments.

lated intracellular signal transduction (46). On this basis, we next examined the involvement of these kinases in PGE_2-stimulated production of Aβ in HEK293 cells, using an inhibitor for each kinase. As shown in Fig. 6, *A* and *B*, neither an inhibitor of PKA, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), nor an inhibitor of PI3K, LY294002, blocked the PGE_2-stimulated production of Aβ. Higher concentrations of these inhibitors (up to 20 μM) gave similar results (data not shown). Furthermore, treatment of cells with both of these inhibitors simultaneously also did not block the PGE_2-stimulated production of Aβ (Fig. 6, *A* and *B*). We also examined the effect of H89 and/or LY294002 on the level of Aβ in the presence of EP_2 agonist and/or EP_4 agonist. As shown in supplemental Fig. S3, neither of these inhibitors affected the level of Aβ in the presence of EP_2 agonist and/or EP_4 agonist in HEK293 cells. These inhibitors, at the concentrations specified in Fig. 6, *A* and *B*, did not affect cell viability (data not shown) and, based on the results of previous publications (47, 48), should have been sufficient to inhibit their target molecules. Therefore, it seems that neither PKA nor PI3K are involved in the PGE_2-stimulated production of Aβ.

Exchange protein directly activated by cAMP (Epac) was recently identified as another target of cAMP for signal transduction (49). We tested the involvement of Epac in PGE_2-stimulated production of Aβ by using a specific activator for Epac, 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′-5′-cyclic monophosphate (pCPT-*O*-Me-cAMP) (49). As shown in Fig. 6, *C* and *D*, pCPT-*O*-Me-cAMP (1–100 μM) did not affect the level of Aβ in the conditioned medium. This chemical at the concentrations specified in Fig. 6, *C* and *D*, did not affect cell viability (data not shown) and, based on previous results (50), should have been sufficient to activate Epac. Therefore, it seems also that Epac is not involved in the PGE_2-stimulated production of Aβ. We confirmed that pCPT-*O*-Me-cAMP did not affect the cell growth at these concentrations used in Fig. 6 (data not shown).

Involvement of APP Phosphorylation and γ-Secretase Activation in PGE_2-stimulated Production of Aβ—We subsequently examined the downstream mechanism for PGE_2-stimulated production of Aβ in HEK293 cells. In general, production of Aβ is regulated by either modification of APP or by secretase activity. At first, we examined the maturation of APP, an essential step in the production of Aβ. The mature (*N*- and *O*-glycosylated) and immature (*N*-glycosylated alone) forms of APP (mAPP and imAPP, respectively) can be separated by SDS-PAGE on the basis of molecular weight (51). As shown in Fig. 7, PGE_2, EP_4 agonist, and EP_4 antagonist (in the presence of PGE_2) did not significantly affect the amounts or ratios of mAPP and imAPP. Consistent with a previous report (44), under similar experimental conditions to those described in Fig. 7, overexpression of endoplasmic reticulum chaperones increased or decreased the levels of imAPP or mAPP, respectively (data not shown). The results shown in Fig. 7 suggest that the PGE_2-

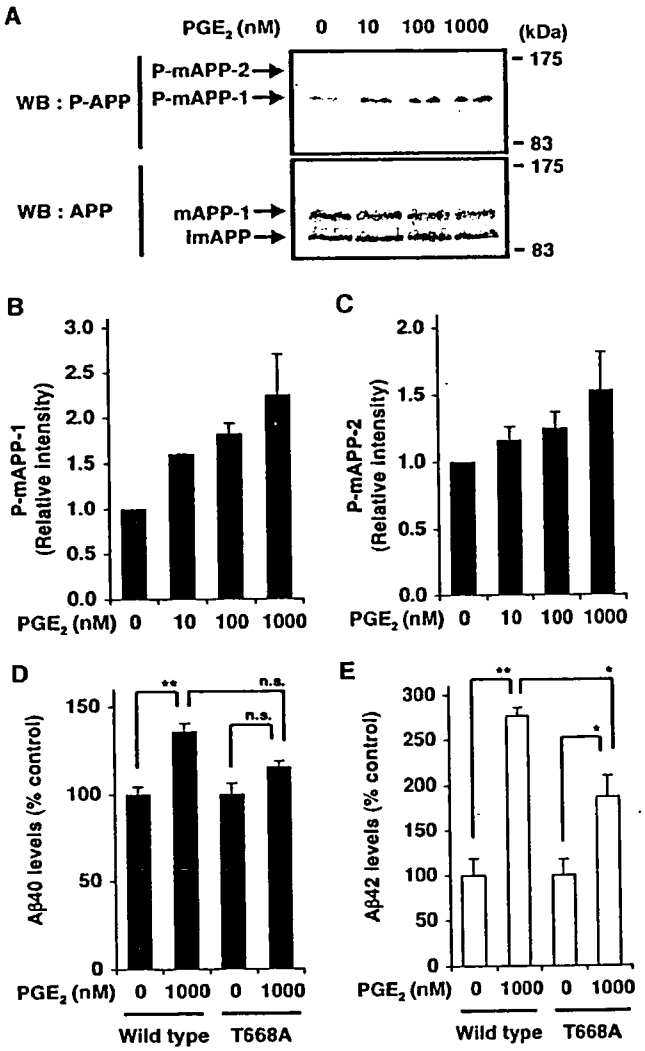

FIGURE 8. **Involvement of the phosphorylation of APP in PGE_2-stimulated production of Aβ.** HEK293 cells expressing APPsw were cultured for 24 h in the presence of the indicated concentrations of PGE_2. Whole cell extracts were analyzed by immunoblotting (*WB*) with an antibody against the C-terminal fragment of APP (*APP*) or APP phosphorylated on Thr-668 (*P-APP*) (*A*). The relative intensity of bands of P-mAPP-1 and P-mAPP-2 in three independent immunoblotting experiments was determined by densitometer (*B* and *C*). HEK293 cells were transiently transfected with expression plasmid for either APP695 (wild type) (pcDNA3/APP695) or APP695 T668A (T668A) (pcDNA3/APP695 T668A). After culture for 24 h, cells were incubated with or without 1000 nM PGE_2 for 24 h. The amounts of Aβ40 and Aβ42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Similar results were obtained in two other independent experiments (*D* and *E*). Values are given as mean ± S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant (*B–E*).

stimulated production of Aβ is not due to any alteration in the maturation of APP. The results also showed that PGE_2 does not increase the total amount of APP (imAPP plus mAPP), thus PGE_2-stimulated production of Aβ cannot be explained by alteration in the amount (expression) of APP by PGE_2.

Phosphorylation of APP, in particular of threonine 668, is another protein modification of APP that may stimulate the production of Aβ (52). We examined the effect of PGE_2 on the level of Thr-668-phosphorylated APP by immunoblotting with an antibody that specifically recognizes this phosphorylated form of APP (Fig. 8*A*). As described previously (52), two bands (P-mAPP-1 and P-mAPP-2) were detected with this antibody and the migration of the lower band (P-mAPP-1) was the same as the band detected by antibody against APP (mAPP-1) (Fig. 8*A*). Although the total amount of the mature or immature forms of APP was not affected by PGE_2, as described in Fig. 7, treatment of cells with PGE_2 increased the amount of Thr-668-phosphorylated APP in a dose-dependent manner but did not affect the total amount of APP (Fig. 8, *A–C*), suggesting that phosphorylation of the Thr-668 of APP is involved in PGE_2-stimulated production of Aβ. To test this possibility, we compared the PGE_2-stimulated production of Aβ in cells expressing wild type APP to those expressing mutant APP (Thr-668 to alanine, T668A). This mutation has been reported to completely suppress the phosphorylation of APP amino acid residue 668 (53). As shown in Fig. 8, *D* and *E*, PGE_2-stimulated production of Aβ was not so distinct in cells expressing mutant APP (T668A) as that observed in cells expressing wild-type APP. Thus, we consider that phosphorylation of APP at Thr-668 is involved in PGE_2-stimulated production of Aβ.

Next, we tested the notion that PGE_2-stimulated production of Aβ is mediated by an alteration of secretase activity. To investigate this possibility, we examined the amount of the CTFs of APP that are generated by α-, β-, or γ-secretase (CTFα, CTFβ, or CTFγ, respectively) (known as an indirect index of secretase activity). As shown in Fig. 9*A*, the amounts of CTFα and CTFβ in cells were not significantly affected by treatment with PGE_2. Furthermore, PGE_2 did not affect the amounts of CTFα and CTFβ even in cells treated with an inhibitor of γ-secretase, DAPT. Treatment of cells with DAPT increased the amount of CTFα and CTFβ (Fig. 9), but did not affect cell growth at the concentration (data not shown). These results suggest that PGE_2 does not affect the activity of α- or β-secretases. CTFγ could not be detected under the same experimental conditions as used for CTFα and CTFβ, as previously described (34). Therefore, we incubated the membrane fractions *in vitro* to stimulate the γ-secretase-mediated proteolysis, as previously described (36). As shown in Fig. 9*B*, a band corresponding to CTFγ was able to be detected and its intensity increased according to the dose of PGE_2, suggesting that PGE_2 activates γ-secretase.

We also examined the effect of EP_4 agonist and EP_4 antagonist on the amounts of the CTFs. As was the case for PGE_2, the EP_4 agonist caused an increase in the intensity of the CTFγ band but not of the bands for CTFα or -β (Fig. 9, *C* and *D*). Furthermore, EP_4 antagonist suppressed the PGE_2-dependent increase in the intensity of the CTFγ band but did not affect the levels of CTFα or -β in the presence of PGE_2 (Fig. 9, *E* and *F*). The results shown in Fig. 9 strongly suggest that PGE_2-stimulated production of Aβ is mediated by activation of γ-secretase but not of α- or β-secretase.

We then directly measured the γ-secretase activity by using an APP-derived fluorescent substrate of γ-secretase (39, 40). As shown in Fig. 10*A*, γ-secretase activity in the solubilized membrane fraction prepared from cells treated with 1000 nM PGE_2 was significantly higher than that from non-treated cells. We confirmed that the addition of 1 μM DAPT or PGE_2 to the assay system inhibited or had no effect, respectively, on γ-secretase activity (data not shown). We also examined the effect of EP_4 agonist and EP_4 antagonist on γ-secretase activity. As was the

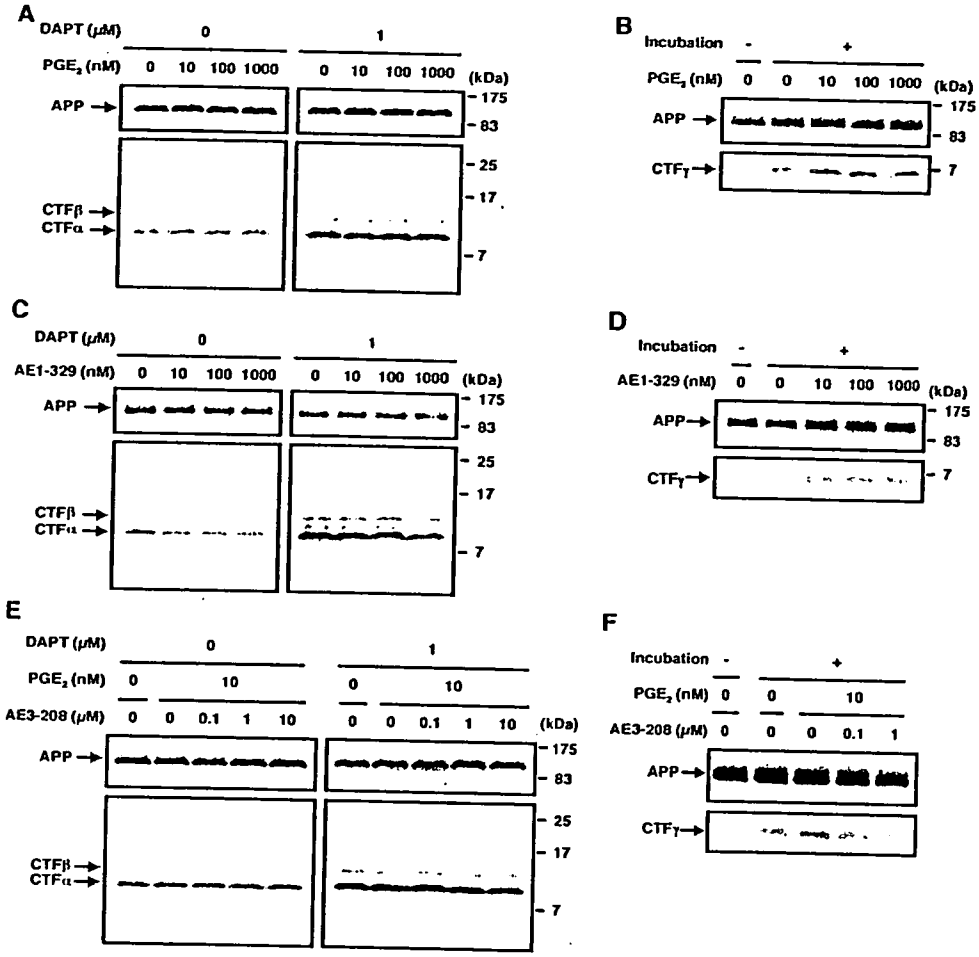

FIGURE 9. **Effect of PGE_2, EP_4 agonist, and EP_4 antagonist on the cellular levels of CTFα, CTFβ, and CTFγ.** HEK293 cells expressing APPsw were cultured for 24 h in the presence of the indicated concentrations of PGE_2 (*A* and *B*), AE1-329 (EP_4 agonist) (*C* and *D*), or PGE_2 plus AE3-208 (EP_4 antagonist) (*E* and *F*) with or without 1 μM DAPT as indicated. Membrane fractions were prepared and half of each was incubated for 2 h at 37 °C. Membrane fractions before (*A*, *C*, and *E*) or after (*B*, *D*, and *F*) the incubation were analyzed by immunoblotting with an antibody against the C-terminal fragment of APP. Similar results in this figure were obtained in two other independent experiments.

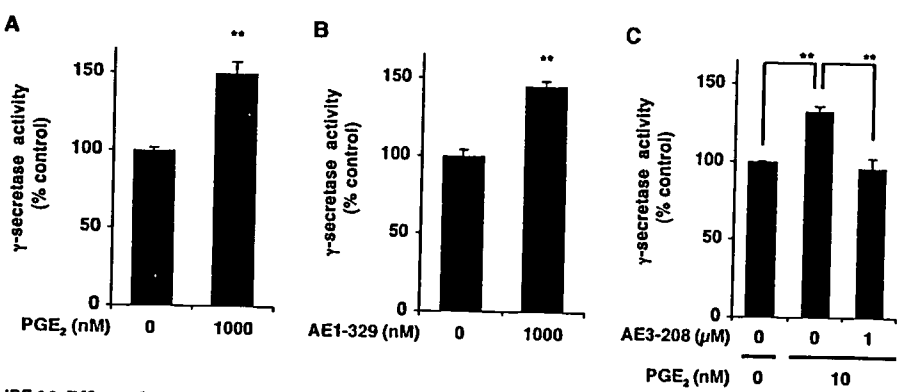

FIGURE 10. **Effect of PGE_2, EP_4 agonist, and EP_4 antagonist on γ-secretase activity.** HEK293 cells expressing APPsw were cultured for 1 h in the presence of the indicated concentrations of PGE_2 (*A*), AE1-329 (EP_4 agonist) (*B*), or PGE_2 plus AE3-208 (EP_4 antagonist) (*C*). Membrane fractions were prepared and subjected to a γ-secretase-mediated peptide cleavage assay as described under "Experimental Procedures." Values are given as mean ± S.E. (n = 3). **, $p < 0.01$. Similar results in this figure were obtained in two other independent experiments.

case for PGE_2, EP_4 agonist activated γ-secretase activity, whereas on the other hand, EP_4 antagonist suppressed the PGE_2-dependent activation of γ-secretase (Fig. 10, *B* and *C*). We also showed that treatment of cells with DAPT inhibited the production of Aβ in the presence or absence of PGE_2 (supplemental Fig. S4). The results presented in Fig. 10 show that treatment of cells with PGE_2 activates γ-secretase in cells.

Involvement of EP_2 and EP_4 Receptors in the Production of Aβ in Vivo—To test the *in vivo* relevance of the *in vitro* results of this study, we examined the effect of deletion of the EP_2 or EP_4 receptor on the level of Aβ in mouse brain. For this purpose, we crossed transgenic mice expressing APPsw (APP23) (30) to $EP_2^{-/-}$ mice (31) or $EP_4^{-/-}$ mice (32) to generate APPsw/$EP_2^{-/-}$ or APPsw/$EP_4^{-/-}$ mice, respectively. These two types of mice developed normally and gained weight at a rate equal to their controls (APPsw/$EP_2^{+/+}$ mice or APPsw/$EP_4^{+/+}$). We measured the amount of Aβ in both soluble and insoluble fractions prepared from mouse brain by sELISA. As shown in Fig. 11, *A–D*, the amount of Aβ in the brains of APPsw/$EP_2^{-/-}$ mice was lower in both fractions than for the APPsw/$EP_2^{+/+}$ mice at the ages of both 3 and 6 months, although the difference in the amounts of Aβ42 in the soluble fraction was not statistically significant at the age of 6 months. On the other hand, the amount of Aβ in the brains of APPsw/$EP_4^{-/-}$ mice was lower in both fractions than that of APPsw/$EP_4^{+/+}$ mice at the age of 6 months, although the difference in the amounts of Aβ42 in the insoluble fraction were not statistically significant (Fig. 11, *G* and *H*). A difference was not as distinct at the age of 3 months (Fig. 11, *E* and *F*). These findings suggest that both EP_2 and EP_4 receptors are also involved in the production of Aβ *in vivo*; in other words, the *in vitro* results obtained in this article are functionally significant. We also compared the level of PGE_2, and mRNA expression of EP_2 and EP_4 receptors between wild-type and APP23 mice. As shown in supplemental Fig. S5, there was no clear difference between them at both 3 and 6 months, except that the amount of PGE_2 was significantly higher in APP23 mice than wild-type mice at the age of 6 months, which is consistent with previous results (54).

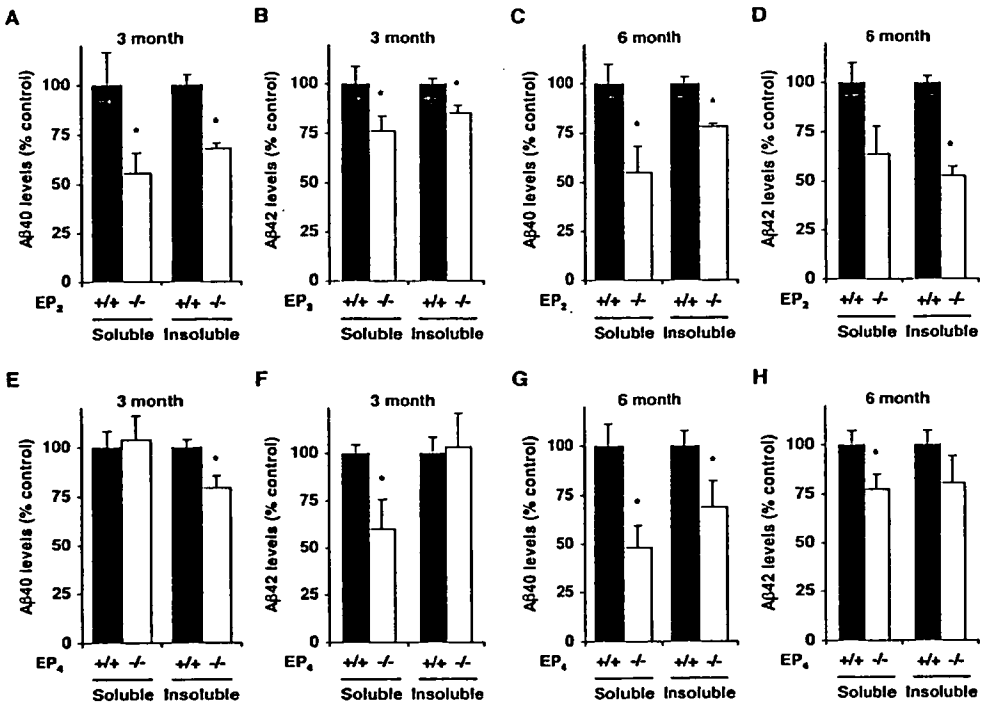

FIGURE 11. **Effects of deletion of the EP_2 or EP_4 receptor on the level of Aβ in the brains of transgenic mice expressing APPsw.** The amounts of Aβ40 and Aβ42 in both the soluble and insoluble fractions prepared from the brains of APPsw/$EP_2^{-/-}$ and APPsw/$EP_2^{+/+}$ mice (*A–D*) or APPsw/$EP_4^{-/-}$ and APPsw/$EP_4^{+/+}$ mice (*E–H*) at the age of 3 and 6 months were determined by sELISA and expressed relative to the control. Values are given as mean ± S.E. (n = 5–6). *, $p < 0.05$. The control values used are (pmol/g tissue) (soluble, insoluble): *A* (0.920 ± 0.154, 8.320 ± 0.445); *B* (0.105 ± 0.010, 1.630 ± 0.048); *C* (1.080 ± 0.109, 14.360 ± 0.506); *D* (0.108 ± 0.015, 2.700 ± 0.221); *E* (1.060 ± 0.086, 8.810 ± 0.315); *F* (0.076 ± 0.004, 1.820 ± 0.329); *G* (1.313 ± 0.149, 15.597 ± 1.206); and *H* (0.142 ± 0.010, 3.030 ± 0.216).

DISCUSSION

It has previously been reported that PGE_2 stimulates production of Aβ in CHO cells (26). In this study, we have confirmed that this also occurs in HEK293 cells and have shown that PGE_2-mediated activation of the EP_4 receptor and the resulting increase in the cellular level of cAMP are responsible for this PGE_2-stimulated production of Aβ. These conclusions have been based on the following observations: EP_4 agonist-stimulated production of Aβ, EP_4 receptor antagonist suppressed the PGE_2-stimulated production of Aβ, an increase in the cellular level of cAMP was observed simultaneously with stimulation of the production of Aβ, and an analogue of cAMP stimulated the production of Aβ. We have also demonstrated that PGE_2 stimulates production of Aβ in SH-SY5Y cells. However, in contrast to what was found for HEK293 cells, our data suggest that both EP_2 and EP_4 receptors are responsible for the PGE_2-stimulated production of Aβ in SH-SY5Y cells. This difference may be due to the fact that the EP_2 receptor is functional in SH-SY5Y but not in HEK293 cells, as suggested previously (55). This suggestion is further supported by our observation that an EP_2 receptor agonist increased the cellular level of cAMP in SH-SY5Y cells but not in HEK293 cells. It is also possible that the low expression of EP_2 receptor in HEK293 cells can explain why this type of cells were inert for the increase in the cellular level of cAMP by the EP_2 agonist. This is the first report of the identification of EP receptors and the intracellular signal transduction pathway for PGE_2-stimulated production of Aβ, which should be important for the development of clinical drugs for AD (see below). Liang *et al.* (56) reported that another EP_2 agonist (butaprost) did not affect the production of Aβ in CHO cells. This seems to be due to the fact that the EP_2 receptor is not functional in CHO cells, as previously described (57), because others have reported that PGE_2 stimulates the production of Aβ in CHO cells (26). A number of previous papers reported that both the EP_2 and EP_4 receptors are expressed in rodent brain and in primary cultures of rodent neurons (58–60). Therefore, it is reasonable to speculate that both EP_2 and EP_4 receptors are functional in rodent (maybe in human) neurons and are responsible for PGE_2-stimulated production of Aβ. Results presented in a previous paper (56) are not consistent with this speculation: they showed that an EP_2 agonist (butaprost) did not affect the production of Aβ in primary cultures of mouse neurons. We speculate that this is because they used primary neurons from mice expressing not only APPsw but also the exon 9-deleted human PS-1 (PS1ΔE9) (see below).

In most of the intracellular signal transduction pathways that are downstream of the increase in the level of cellular cAMP, both PKA and PI3K play important roles (46). However, results from experiments using kinase inhibitors suggest that neither of these kinases are involved in PGE_2-stimulated production of Aβ. Furthermore, results from experiments using a specific activator for Epac suggested that Epac is also not involved in PGE_2-stimulated production of Aβ. Therefore, an unknown mechanism, located downstream of the cAMP increase, seems to be involved in the signal transduction pathway for PGE_2-stimulated production of Aβ.

It was previously reported that PGE_2 increases the amount of CTFγ in CHO cells (26); however, the molecular mechanism governing this increase remained unknown. In this study, we observed that not only PGE_2, but also an EP_4 agonist, increased CTFγ levels in HEK293 cells. Furthermore, we directly measured γ-secretase activity and showed that the activity was higher in cells treated with PGE_2 or EP_4 agonist than in control cells. These results suggest that activation of γ-secretase is responsible for both the PGE_2-mediated increase in the cellular level of CTFγ and for stimulation of the production of Aβ. Because overexpression of PS1ΔE9 has been shown to cause activation of γ-secretase (61, 62), we consider that the higher activity of γ-secretase in cells expressing PS1ΔE9 may mask the EP_2-mediated activation of

γ-secretase and this could explain why, in the previous report (56), the EP_2 agonist butaprost did not affect the production of Aβ. We also found that phosphorylation of Thr-668 in APP is stimulated in cells treated with PGE_2 and that cells expressing mutant APP (T668A) showed less of a response than the wild type control against PGE_2-mediated stimulation of Aβ production. This suggests that Thr-668 phosphorylation of APP is also involved in PGE_2-stimulated production of Aβ. However, the molecular mechanisms whereby the increase in the cellular level of cAMP affects phosphorylation of Thr-668 of APP remains unknown. A number of previous reports suggested that the γ-secretase activity is modulated by various molecules such as phospholipase D1, CD147, and platelet-derived growth factor (63–65), therefore, it is possible that the increase in the level of cellular cAMP affect γ-secretase activity through modulation of activities of these molecules.

In this paper, both EP_2 and EP_4 receptors have been shown to be involved in the production of Aβ not only *in vitro* but also *in vivo*: the amount of Aβ in the brains of APPsw/$EP_2^{-/-}$ and APPsw/$EP_4^{-/-}$ mice was lower than in control mice. This is the first demonstration that deletion of the EP_4 receptor decreases the level of Aβ *in vivo* and we speculate that this decrease is due to the lack of EP_4-mediated activation of γ-secretase by PGE_2, as was seen *in vitro*. Liang *et al.* (56) recently reported that deletion of the EP_2 receptor, in the genetic background of mice expressing both APPsw and PS1ΔE9, decreased the level of Aβ. They suggested that this decrease is due to loss of the EP_2 receptor in microglial cells where they are responsible for the production of reactive oxygen species, which activate β-secretase in neurons. Deletion of the EP_2 receptor decreased the level of Aβ at the age of 3 months in our mouse model (without expression of PS1ΔE9) but this effect was observed at ages greater than 8 months in their mouse model (with the expression of PS1ΔE9). We speculate that the higher activity of γ-secretase caused by the expression of PS1ΔE9 masks the effect of the deletion of the EP_2 receptor on γ-secretase activity that can be seen at younger ages in mice that do not express PS1ΔE9. Based on these observations, we consider that the EP_2-mediated direct activation of γ-secretase or activation of β-secretase via reactive oxygen species contributes to the alteration in the level of Aβ in the brain of EP_2 knock-out mice at younger or older ages, respectively. In other words, the EP_2 receptor is involved in the production of Aβ through at least two independent mechanisms.

As described above, NSAIDs have attracted much attention as a new class of drugs for the treatment and prevention of AD, although some animal and clinical studies showed negative results for the advantage of NSAIDs as drugs for AD (19, 66, 67). Although some reports suggest that the COX-independent actions of NSAIDs (such as direct binding to γ-secretase, activation of the peroxisome proliferators activated receptor-γ, and resulting inhibition of β-secretase, inhibition of Rho/Rho kinase (Rock) pathway, and activation of nuclear factor-κB (NF-κB)) are involved in the inhibitory effect of NSAIDs on the production of Aβ (19, 21, 22, 68), COX inhibition, and the resulting decrease in the level of PGE_2 seem to play an important role in the anti-AD activity of NSAIDs (26, 69). COX exists as two subtypes, COX-1 and COX-2, and NSAIDs can be classified into two groups: newly developed COX-2-specific NSAIDs (such as celecoxib) and classical NSAIDs without COX-2 specificity (such as indomethacin). Clinical use of classical NSAIDs is associated with gastrointestinal side effects (23), because PGs have a strong protective effect on gastrointestinal mucosa (70, 71). Because COX expressed in gastrointestinal mucosa is mainly COX-1, COX-2-specific NSAIDs have less of an effect on the levels of PGs at gastrointestinal mucosa and therefore, would cause less gastrointestinal side effects than classical NSAIDs. However, it was recently shown that clinical use of COX-2-specific NSAIDs is associated with cardiovascular thrombotic side effects (24, 25). This is because prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 in vascular endothelial cells, whereas thromboxane A_2, a potent aggregator of platelets and a vasoconstrictor, is mainly produced by COX-1 in platelets (72, 73). These side effects of NSAIDs would most likely become problematic when used long-term for the prevention or treatment of AD. On the other hand, results of this study suggest that antagonists for either the EP_2 or EP_4 receptors, or more importantly antagonists for both EP_2 and EP_4 receptors, will be effective for the treatment and prevention of AD through inhibiting the production of Aβ. EP_1 and EP_3 receptors were reported to be involved in PGE_2-mediated protection of gastrointestinal mucosa through stimulating the production of bicarbonate and gastric mucosal blood flow, respectively (74, 75). Therefore, antagonists specific for both EP_2 and EP_4 would be safer for gastrointestinal mucosa than NSAIDs. At present, there are no reports of specific EP_2 antagonists and it is unclear whether or not the EP_4 antagonist used in this study (AE3-208) is able to cross the blood-brain barrier. Therefore, we predict that blood-brain barrier-permeable antagonists for both EP_2 and EP_4 receptors will be therapeutically beneficial for AD. Of course, it should be noted that this type of drugs may not be as effective as NSAIDs, if the COX-independent actions of NSAIDs (see above) are predominant for their anti-AD actions.

Acknowledgment—We thank Dr. M. Staufenbiel (Novartis Pharma Ltd.) for providing the APP23 mice.

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