# Apolipoprotein A-I induces translocation of protein kinase Cα to a cytosolic lipid-protein particle in astrocytes

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**Abstract** **Apolipoprotein A-I (apoA-I) induces the translocation of newly synthesized cholesterol as well as caveolin-1 to the cytosolic lipid-protein particle (CLPP) fraction in astrocytes before its appearance in high density lipoprotein generated in the medium (Ito, J., Y. Nagayasu, K. Kato, R. Sato, and S. Yokoyama. 2002. Apolipoprotein A-I induces translocation of cholesterol, phospholipid, and caveolin-1 to cytosol in rat astrocytes. *J. Biol. Chem.* 277: 7929–7935). We here report the association of signal-related molecules with CLPP. ApoA-I induces rapid translocation of protein kinase Cα to the CLPP fraction and its phosphorylation in astrocytes. ApoA-I also induces the translocation of phospholipase Cγ to CLPP. Diacylglyceride (DG) production is increased by apoA-I in the cells, with a maximum at 5 min after the stimulation, and the increase takes place also in the CLPP fraction. An inhibitor of receptor-coupled phospholipase C, U73122, inhibited all the apoA-I-induced events, such as DG production, cholesterol translocation to the cytosol, release of cholesterol, and translocation of protein kinase Cα into the CLPP fraction. CLPP may thus be involved in the apoA-I-initiated signal transduction in astrocytes that is related to intracellular cholesterol trafficking for the generation of high density lipoprotein in the brain.**—Ito, J-i., H. Li, Y. Nagayasu, A. Kheirollah, and S. Yokoyama. **Apolipoprotein A-I induces translocation of protein kinase Cα to a cytosolic lipid-protein particle in astrocytes.** ***J. Lipid Res.*** **2004.** 45: **2269–2276.**

**Supplementary key words** caveolin-1 • phospholipase C • phosphatidylinositol turnover • cholesterol

The main apolipoproteins in mammalian cerebrospinal fluid (CSF) are apolipoprotein A-I (apoA-I) and apoE (1–3), which are present as HDL and play major roles in intercellular cholesterol transport in the brain (4), being segregated by the blood-brain barrier from the lipoprotein system in the systemic circulation. Astrocytes and partly microglia cells generate cholesterol-rich HDL by endogenous apoE along with cellular cholesterol and phospholipid (5–9). These HDLs may transport cholesterol to the neural cells where it is required via the cellular receptors that recognize lipid-bound apoE (10). ApoE-HDL was indeed shown to play a critical role in wound healing of the brain (11). ApoA-I is also found in human CSF as the second major apolipoprotein, with a concentration almost equivalent to that of apoE (12–14), but the source of this protein is unclear. No neural cell is believed to produce apoA-I, whereas the brain capillary endothelial cells produce apoA-I, although it is uncertain whether it is secreted into the CSF (15, 16). Some authors propose that the apoA-I in the systemic circulation is transported across the blood-brain barrier (3, 4).

In addition to the production of apoE-HDL, astrocytes interact with exogenous apoA-I to generate phospholipid-rich and cholesterol-poor HDL (5, 17, 18). The physiological relevance of this observation in human brain has been supported by the facts that the apoA-I concentration in CSF is high enough to carry this reaction (13, 14) and that apoA-I dissociates from HDL to interact with the cells (19). The cholesterol-rich apoE-HDL and cholesterol-poor apoA-I-HDL may play differential roles in intercellular cholesterol transport in the brain.

In a previous paper, we demonstrated transient translocation of newly synthesized cholesterol and phospholipid to the cytosol from the endoplasmic reticulum and Golgi apparatus when exogenous apoA-I interacted with rat astrocytes and generated HDL (17, 20, 21). Transient translocation of caveolin-1 to the cytosol was also induced in a similar time-dependent manner to the lipid translocation (20). The lipids and caveolin-1 in the cytosol were recovered along with cyclophilin A in the cytosolic fraction, having the same density as plasma HDL [cytosolic lipid-protein particle (CLPP)]. The CLPP is a particle composed of proteins and lipids such as cholesterol, sphingomyelin,

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Abbreviations: apoA-I, apolipoprotein A-I; apoE-KO mouse, apoE knockout C57BL/6 mouse; CLPP, cytosolic lipid-protein particle; CSF, cerebrospinal fluid; DG, diacylglyceride; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; PI, phosphatidylinositol.

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and phosphatidylcholine with a diameter of 17–18 nm and a density of 1.08–1.12 g/ml (20). Cyclosporin A, a cyclophilin A inhibitor, inhibited this apoA-I-induced translocation and also apoA-I-mediated cholesterol release. Caveolin-1 is believed to play an important role in intracellular cholesterol trafficking, so that it is rational to hypothesize that CLPP is involved in the intracellular cholesterol transport stimulated by extracellular apoA-I for the generation of HDL. We attempted to investigate potential signaling pathways in astrocytes for apoA-I to stimulate lipid trafficking in relation to the function of CLPP. Protein kinase Cα and its related signaling molecules were found associated with this particle when cells were stimulated by apoA-I.

## MATERIALS AND METHODS

### Materials

ApoA-I was prepared from freshly isolated human HDL by delipidation and anion-exchange chromatography according to the method described elsewhere (22). ApoE was prepared from hyperlipidemic human plasma as previously described (23). Inhibitors of receptor-coupled phospholipase C and its inactive analog, U73122 and U73343 (24), were purchased from WAKO Pure Chemical.

### Cell culture

Astrocytes were prepared according to the method previously described from the cerebrums of 17 day old fetal Wistar rat (25), C57BL/6 mouse, and apoE knockout C57BL/6 mouse (apoE-KO mouse) purchased from Taconic/IBL (Germantown, NY/Fujioka,

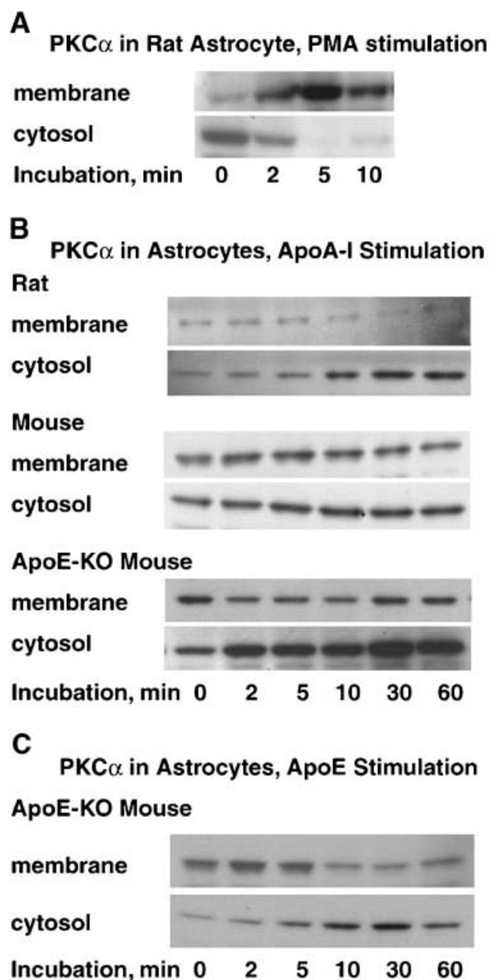



**Fig. 1.** Redistribution by apolipoprotein A-I (apoA-I) of protein kinase Cα (PKCα) in astrocytes. A: Rat astrocytes were treated with 200 nM phorbol 12-myristate 13-acetate (PMA). The membrane fraction protein (15 μg/lane) and the cytosol protein (50 μg/lane) were analyzed for protein kinase Cα by immunoblotting. Translocation of protein kinase Cα was demonstrated from the cytosol to the membrane. B: Astrocytes of rat, mouse, and apoE knockout C57BL/6 mouse (apoE-KO mouse) were incubated with 5 μg/ml apoA-I for the indicated period of time in 0.02% BSA/F-10, 0.02% BSA/DMEM, and 0.02% BSA/DMEM, respectively. The cytosol protein (30 μg/lane) and the membrane protein (15 μg/lane) were analyzed for protein kinase Cα. C: Astrocytes of an apoE-KO mouse were incubated with 5 μg/ml apoE. The same analysis was performed for protein kinase Cα.

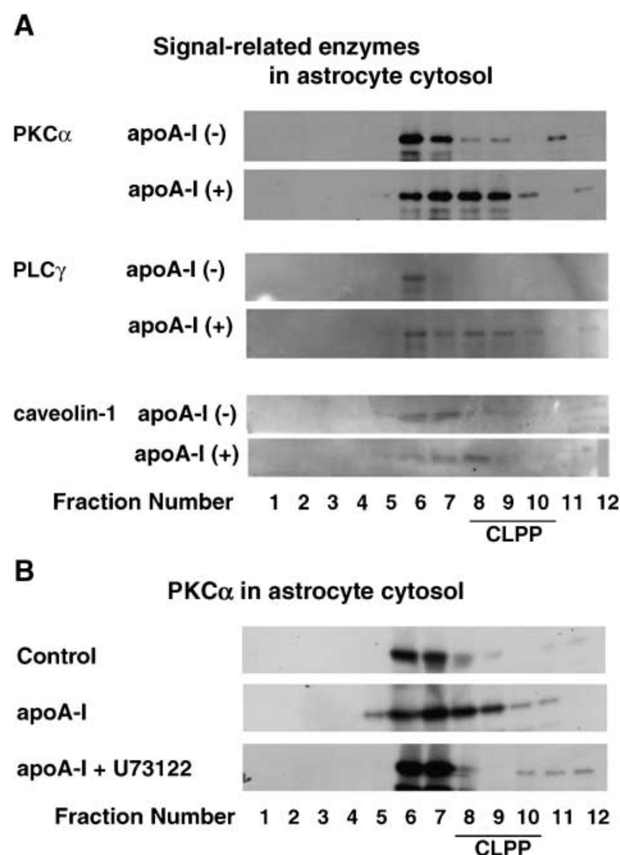



**Fig. 2.** Redistribution by apoA-I of protein kinase Cα (PKCα) and phospholipase Cγ (PLCγ) in cytosol of mouse astrocytes. A: After washing and medium replacement with 0.02% BSA/DMEM, apoE-KO mouse astrocytes were incubated with or without apoA-I (5 μg/ml) for 5 min. The cytosol (350 μg protein/7 ml) was prepared from the cells and centrifuged on the sucrose solution (18 ml) with a density of 1.17 g/ml at 49,000 rpm for 48 h and separated into 12 fractions from the bottom. Protein was precipitated with 10% TCA and analyzed by SDS-PAGE and Western blotting using rabbit anti-protein kinase Cα, mouse anti-phospholipase Cγ, and rabbit anti-caveolin-1 antibodies. CLPP, cytosolic lipid-protein particle. B: The cytosol (380 μg/7 ml) was prepared from apoE-KO mouse astrocytes treated with apoA-I (0 or 5 μg/ml) for 5 min with or without a 5 min pretreatment with 10 μM U73122. The cytosol was centrifuged as described in A and separated into 12 fractions from the bottom. The 10% TCA-precipitated protein of each fraction was analyzed by SDS-PAGE and Western blotting using rabbit anti-protein kinase Cα.

Japan). After removal of the meninges, the cerebral hemisphere was cut into small pieces and treated with 0.1% trypsin solution in Dulbecco's phosphate-buffered saline (DPBS) containing 0.15% glucose (0.1% trypsin/DPBS/G) for 3 min at room temperature. The cell pellets obtained by centrifugation at 1,000 rpm for 3 min were cultured in F-10 medium containing 10% fetal calf serum (10% FCS/F-10) for rat astrocytes or 15% FCS/DMEM for mouse astrocytes at 37°C for 1 week. The cells were treated with 0.1% trypsin/DPBS/G containing 1 mM EDTA again and then cultured in 10% FCS/F-10 or 15% FCS/DMEM using a six-well multiple tray for 1 week. Human fibroblast cell line WI-38 cells (RIKEN Cell Bank) were grown in 10% FCS/DMEM.

### Cytosol preparation and density gradient ultracentrifugation analysis

Cytosol of astrocytes was prepared according to the method of Thom et al. (26). Cell pellet was obtained by centrifugation at 1,000 rpm for 10 min after washing the cells with DPBS four times and harvesting them with a rubber policeman. The pellet was treated with cold 0.02 M Tris-HCl buffer, pH 7.5 containing a protease inhibitor cocktail (Sigma) for 15 min, with 10 s of strong agitation (25 times) every 5 min. The cell suspension was centrifuged at 2,000 *g* for 20 min for preparation of the denuclear-supernatant fraction, and the supernatant was centrifuged at 367,000 *g* for 30 min at 4°C to obtain a cytosol fraction. The cytosol (7 ml) was overlaid on top of the sucrose solution at the density of 1.17 g/ml (18 ml) and centrifuged at 49,000 rpm for 48 h at 4°C using a Hitachi RP50T rotor. The solution in the centrifuge tube was collected from the bottom into 12 fractions.

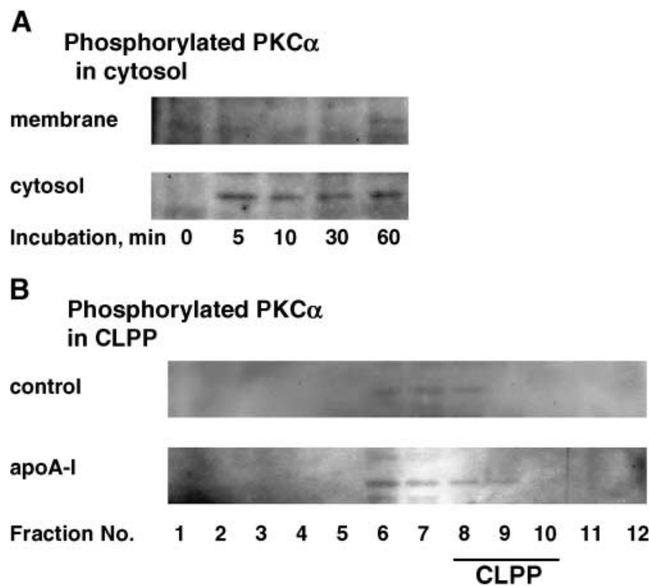



**Fig. 3.** Phosphorylation of protein kinase Cα (PKCα) in apoA-I-stimulated mouse astrocytes. A: The cytosol and membrane fractions were prepared from mouse astrocytes pretreated with 5 μg/ml apoA-I for the indicated periods of time in fresh 0.02% BSA/DMEM. Each sample was analyzed by SDS-PAGE (40 μg/lane for the cytosol fraction and 25 μg/lane for the membrane fraction) and Western blotting using goat anti-phospho-protein kinase Cα at residue serine-657 (Santa Cruz Biotechnology). B: The cytosol fraction (267 μg/7 ml) from the cells pretreated with or without apoA-I (5 μg/ml) for 5 min was centrifuged at 49,000 rpm for 48 h on 1.174 g/ml sucrose solution (18 ml) and separated into 12 fractions. Each fraction was analyzed by SDS-PAGE and Western blotting using goat anti-phospho-protein kinase Cα at residue serine-657 after precipitation with 10% TCA.

### Caveolae/rafts preparation from the membrane fraction

The membrane fraction was prepared by centrifugation at 17,000 *g* for 60 min or 367,000 *g* for 30 min from the denuclear-supernatant fraction. The membrane pellet in 0.75 ml of 0.02 M Tris-HCl buffer containing a protease inhibitor cocktail was sonicated six times every 10 s at level 6 with a Taitec UP-55 homogenizer. After adjustment of the membrane solution to 30% sucrose by adding 0.75 ml of 60% sucrose solution and mixing, 1.5 ml of 10% sucrose solution was overlaid, followed by centrifugation at 367,000 *g* for 60 min. The sample was collected from the bottom of the centrifugation tube into five fractions and analyzed by SDS-PAGE (0.5% SDS/12.5% polyacrylamide gel). The caveolae/rafts fraction was recovered as fraction 3.

### Western blotting

The membrane fraction was prepared and sonicated in 0.02 M Tris-HCl buffer, pH 7.5, containing protease inhibitor cocktail (Sigma). Protein was precipitated by centrifugation at 15,000 rpm for 20 min in the presence of 10% TCA from cytosol or the sonicated membrane fraction. The resolubilized protein pellet was applied to SDS-PAGE and transferred to a Sequi-Blot™ polyvinylidene fluoride membrane (Bio-Rad). The membrane was immunostained with rabbit anti-protein kinase Cα (Sigma), mouse anti-phospholipase Cγ (BD Transduction Laboratories), rabbit anti-caveolin-1 (Santa Cruz Biochemistry), and goat anti-phospho-PKCα (Ser-657) (Santa Cruz Biochemistry) antibodies.

### De novo syntheses and release of lipid

Astrocytes at a confluent cell density were washed with DPBS four times and incubated in 0.1% BSA/F-10 for rat astrocytes or 0.1% BSA/DMEM for mouse astrocytes and WI-38 cells for 24 h. To measure de novo syntheses and release of cholesterol and phospholipid, the cells were incubated with [$^3$H]acetate (20 μCi/ml; New England Nuclear) in fresh 0.02% BSA/F-10 or 0.02% BSA/DMEM for various periods of time. After the cells were washed three times with cold DPBS, lipid was extracted from the cells or from the conditioned medium with hexane-isopropanol (3:2, v/v) solvent mixture or chloroform-methanol (2:1, v/v) mixture, respectively, and analyzed by TLC on Silica Gel-60 plates (E. Merck, Darmstadt, Germany) according to the method previously described (27). The cells were incubated with [$^3$H]acetate (20 μCi/ml) or [$^{14}$C]glycerol (0.2 μCi/ml; Amersham Biosciences) for various periods of time. The diacylglyceride (DG) was extracted from the cells, followed by TLC with diethylether-benzene-ethanol-acetic acid (200:250:10:1, v/v) solvent (16).

TABLE 1. Increase of DG production by apoA-I in mouse astrocytes

| Apolipoprotein | Membrane | Cytosol | Total |
|---|---|---|---|
| ApoA-I (−) | 23,451 ± 607 | 4,237 ± 154 | 27,688 ± 761 |
| ApoA-I (+) | 20,692 ± 1,159 | 15,708 ± 369 | 36,400 ± 790 |

apoA-I, apolipoprotein A-I; DG, diacylglyceride. Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [$^3$H]acetate in 1 ml of 0.02% BSA/DMEM. After washing and medium replacement with fresh 0.02% BSA/DMEM, the cells were incubated with or without 5 μg/ml apoA-I for 5 min. The denuclear-supernatant fraction was prepared as described in Materials and Methods. The cytosol and total membrane fractions were prepared by centrifugation at 367,000 *g* for 30 min as the supernatant and the pellet, respectively. Lipid was extracted from the total membrane fraction (62 μg of protein) and the total cytosol (347 μg/7 ml), and radioactivity in DG was determined after separation by TLC according to the method described in Materials and Methods. Each value represents the average and SEM of triplicate samples in total dpm.

## RESULTS

When rat astrocytes were stimulated with 200 nM phorbol 12-myristate 13-acetate, protein kinase Cα was translocated from the cytosol to the membrane fraction (**Fig. 1A**). To our surprise, however, apoA-I induced the translocation of protein kinase Cα in the reverse direction, from the membrane to the cytosol fraction, in the astrocytes prepared from rats, wild-type mice, and apoE-KO mice, at 2–10 min after stimulation (Fig. 1B). The effect of apoA-I was smaller in wild-type mice than in apoE-KO mice, perhaps because of baseline autocrine stimulation by apoE in the former cells. This was confirmed by the effect of apoE on the cells of an apoE-KO mouse to demonstrate the similar translocation of protein kinase Cα to that by apoA-I (Fig. 1C). This result also indicated that the reaction is not apoA-I-specific and seems helical apolipoprotein-specific. A small increase of the membrane-bound enzyme was observed by long-term incubation in the apoE-KO cells for an unknown reason.

The cytosol was analyzed by density gradient ultracentrifugation for change in the distribution of protein kinase Cα after the 5 min stimulation by apoA-I in apoE-KO mouse astrocytes, because the increase of protein kinase Cα by apoA-I was most prominent in this type of cell. **Figure 2A** demonstrates that protein kinase Cα increased in the CLPP fractions (fractions 8–10) by apoA-I stimulation for 5 min. Interestingly, phospholipase Cγ also increased in the same fraction at 5 min after apoA-I stimulation. Caveolin-1 was recovered in this fraction and apoA-I caused its further increase, consistent with our previous findings with rat astrocytes (20). The increase of protein kinase Cα in the CLPP fraction was reversed by a receptor-coupled phospholipase C inhibitor, U73122 (Fig. 2B). Faint bands of protein kinase Cα were also observed in the lower density fractions of the control cells and the U73122-treated cells. These fractions are to be investigated further.

It is an important question whether protein kinase Cα is activated when it is translocated to CLPP by apoA-I stimulation. The activity of protein kinase Cα is reportedly associated with its phosphorylation at the serine-657 residue (28). The phosphorylated enzyme was probed by a specific antibody, and it increased in the astrocyte cytosol of apoE-KO mouse after the 5 min stimulation by apoA-I (**Fig. 3A**). When the cytosol was analyzed by density gradient ultracentrifugation, the phosphorylated protein kinase Cα was increased in the CLPP fractions (fractions 8–10), although a major portion of the phosphorylated enzyme was in the heavier fraction (fractions 6 and 7) (Fig. 3B).

As apoA-I may initiate signal transduction, the production of DG was monitored in mouse astrocytes when apoA-I was added to the medium (5 μg/ml) (**Table 1**). DG pro-

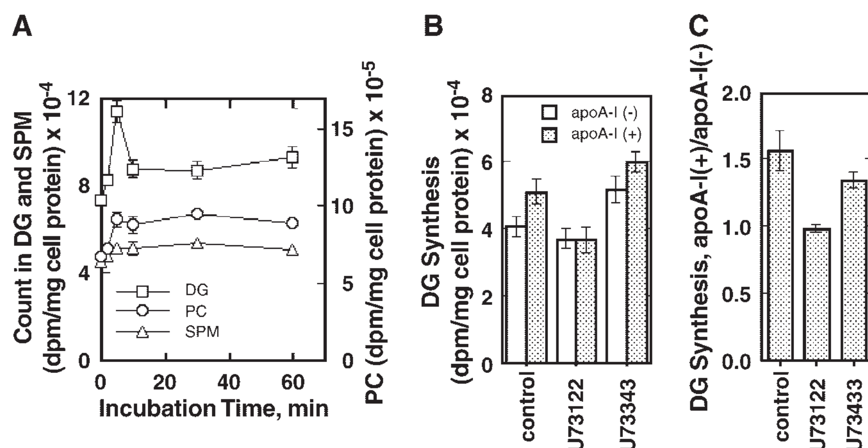



**Fig. 4.** Increase of diacylglyceride (DG) production by apoA-I and the effect of U73122 in mouse astrocytes. A: Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [$^3$H]acetate in 1 ml of DMEM medium containing 0.02% BSA (0.02% BSA/DMEM). After three complete washes with Dulbecco's phosphate-buffered saline containing 0.15% glucose (DPBS/G), the cells were incubated for 60 min in fresh 0.02% BSA/DMEM. ApoA-I (5 μg/ml) was added to the medium at 0, 30, 50, 55, 58, and 60 min after the start of the incubation, to make the incubation periods with apoA-I 60, 30, 10, 5, 2, and 0 min. Lipid was then extracted with hexane-isopropanol (3:2) from the whole cells and separated by TLC. Radioactivity was determined for DG, sphingomyelin (SPM), and phosphatidylcholine (PC). Each data point represents the average and SEM of triplicate samples. B: Rat astrocytes were treated with (dotted columns) or without (open columns) apoA-I (5 μg/ml) in 0.02% BSA/F-10 in the presence or absence of U73122 (10 μM) or U73343 (10 μM) for 2 h. After three washes with DPBS, the cells were incubated for 1 h with 0.2 μCi/ml [$^{14}$C]glycerol in fresh 0.02% BSA/F-10. Lipid was extracted from the cells and separated by TLC. Radioactivity was determined for DG. Each data point represents the average and SEM of triplicate samples. C: Mouse astrocytes were pulse-labeled for 3 h with 20 μCi/ml [$^3$H]acetate in 0.02% BSA/DMEM and washed three times with DPBS. The cells treated with U73122 (10 μM) or U73343 (10 μM) in 0.02% BSA/DMEM for 30 min were incubated with apoA-I (0.5 μg/ml) for 5 min. After washing, lipids were extracted from the cells and analyzed by TLC, and radioactivity was determined for DG. The data are expressed as the ratio of DG synthesis with apoA-I against that without apoA-I. Data represents mean ± SE for three measurements.

duction transiently increased at 5 min of incubation with apoA-I (**Fig. 4A**). This is distinct from the sphingomyelin replenishment reaction to generate DG with respect to the time course (21). This rapid and transient increase of DG implied the involvement of phosphatidylinositol (PI) turnover and the activation of phospholipase Cγ. This view was supported by the finding that U73122 suppressed the increase of DG production by apoA-I but U73343, an inactive analog of U73122, did not (Fig. 4B, C). These findings were also identical in human fibroblast WI-38 (**Fig. 5**). The site of this DG increase was analyzed in mouse astrocytes (**Fig. 6**). DG in the membrane fraction was mainly localized in the caveolin-1-rich caveolae/rafts fraction and did not show significant change by apoA-I stimulation (Fig. 6A). On the other hand, cholesterol and phosphatidylcholine in the cytosol were recovered in the fraction at a density of 1.07–1.12 g/ml (CLPP) (Fig. 6B). Unlike our previous finding in rat astrocytes under stimulation by apoA-I for 90 min (20), treatment of the cells with apoA-I for 5 min was not long enough to cause significant translocation of cholesterol and phospholipid to this fraction. However, apoA-I induced the increase of DG in this fraction by 5 min incubation (Fig. 6C). U73122 canceled the apoA-I-induced cholesterol translocation to the cytosol and its release by apoA-I (**Fig. 7**).

## DISCUSSION

We recently reported that exogenous apoA-I induces the transient translocation of caveolin-1 and newly synthesized cholesterol to CLPP that also contains cyclophilin A in rat astrocytes (20). As many previous reports indicated that helical apolipoproteins, especially apoA-I, initiate intracellular signal transduction (29, 30), it is important to clarify whether this cholesterol translocation is induced by a specific signal(s) or by other mechanism such as a metabolic cascade triggered by the removal of lipid by apolipoprotein (31). We here investigated the association of signal-relating molecules with CLPP induced by apoA-I in astrocytes, indicating the potential involvement of this particle in signal transduction to mobilize cholesterol for the generation of HDL.

The results are summarized as follow. *1*) ApoA-I rapidly induced the translocation of phospholipase Cγ and protein kinase Cα to the CLPP fraction, and the latter was phosphorylated. The translocation of protein kinase Cα was inhibited by a receptor-coupled phospholipase C inhibitor, U73122. *2*) DG transiently increased by apoA-I at the 5 min incubation, and this increase was suppressed by U73122. The increase of DG was not observed in the membrane fraction but in the CLPP fraction. *3*) U73122 also suppressed both the apoA-I-mediated cholesterol release and related changes in cholesterol metabolism, such as cholesterol translocation to the cytosol.

These findings are consistent with the view that apoA-I initiates rapid signal transduction by receptor-coupled phospholipase C-mediated DG production, presumably through a PI turnover pathway. In most of the initiation of signal transduction, the activation of phospholipase Cγ occurs through the interaction of its SH-2 domain with a receptor that is tyrosine-autophosphorylated by binding a specific ligand, and DG is generated in the plasma membrane through the enhancement of PI turnover (32). Therefore, activation of the signaling pathway is associated with translocation of the signal-related enzymes from the cytosol to the membrane. To our surprise, apoA-I induced the translocation of phospholipase Cγ from the membrane to the cytosol in astrocytes. Further analysis of the cytosol revealed that the increase was in the CLPP fraction, and the increase of DG also takes place in this fraction rather than in the membrane. It is still unknown whether phospholipase Cγ is translocated to CLPP after its activation in the plasma membrane or is activated in the CLPP after the translocation. We were unable to detect the tyrosine-phosphorylated phospholipase Cγ in CLPP (data not shown). Nevertheless, it appears reasonable to assume that DG is generated in the CLPP fraction by the phospholipase Cγ translocated to this fraction. At present, we do not know the mechanisms by which phospholipase Cγ is translocated to CLPP and its activation. Phospholipase Cγ has a pleckstrin homology domain to bind PI 4,5-bisphosphate selectively (33). If PI turnover is triggered to produce this molecule in the CLPP by apoA-I stimulation, phospholipase Cγ may then be translocated to the CLPP. Also, we

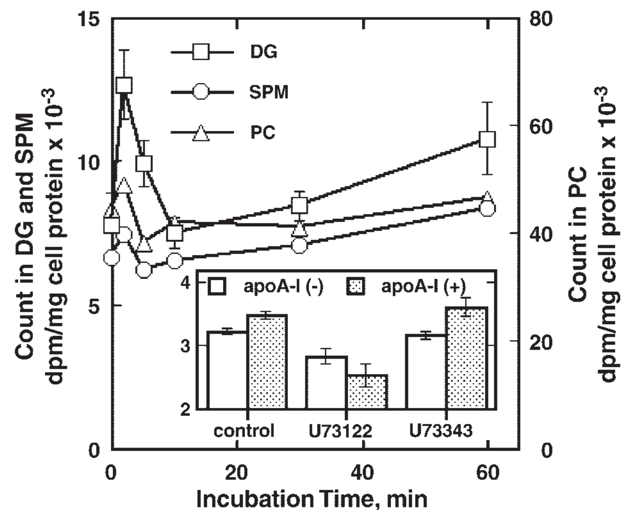



**Fig. 5.** Increase of DG production by apoA-I and the effect of U73122 on DG production in WI-38 cells. WI-38 cells were pulse-labeled for 3 h with 20 μCi of [$^3$H]acetate in 1 ml of 0.02% BSA/DMEM. The cells were incubated with apoA-I (5 μg/ml) for 0, 2, 5, 10, 30, and 60 min as described for Fig. 4A. Lipid was then extracted from the whole cells and separated by TLC for the determination of DG, sphingomyelin (SPM), and phosphatidylcholine (PC). Each data point represents the average and SEM of triplicate samples. In the inset, WI-38 cells were treated with (dotted columns) or without (open columns) apoA-I (5 μg/ml) in 0.02% BSA/DMEM in the presence or absence of U73122 (10 μM) or U73343 (10 μM) for 2 h. After washing three times with DPBS, the cells were incubated for 1 h with 20 μCi/ml [$^3$H]acetate in fresh 0.02% BSA/DMEM with or without U73122 or U73433. Lipid was extracted from the cells and separated by TLC for DG determination. Each data point represents the average and SEM of triplicate samples.

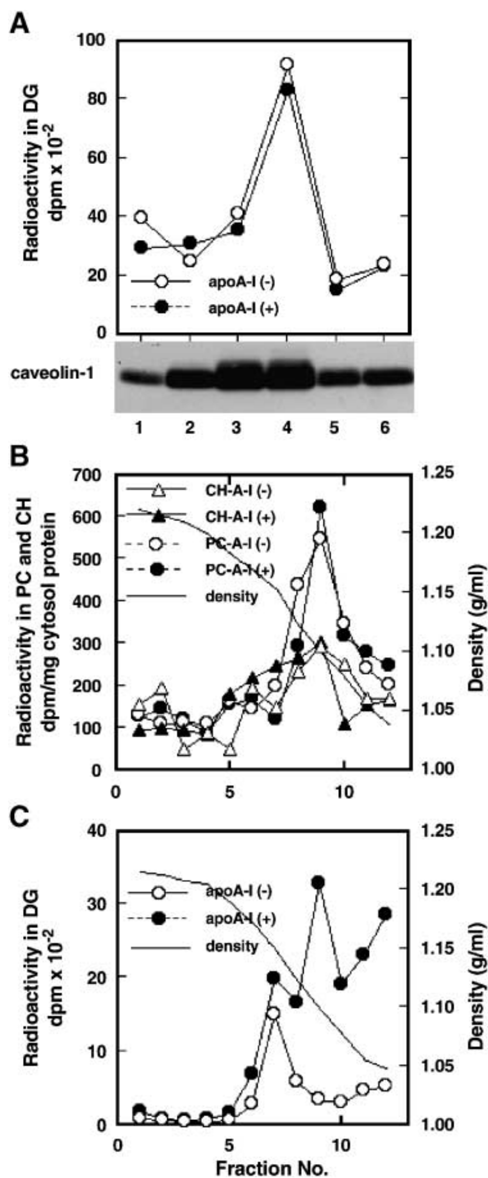



**Fig. 6.** Increase of DG by apoA-I in the cytosol of astrocytes. Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [$^3$H]acetate in 1 ml of 0.02% BSA/DMEM and then treated with (closed symbols) or without (open symbols) 5 μg/ml apoA-I for 5 min after washing and medium replacement with fresh 0.02% BSA/DMEM. The denuclear-supernatant fraction was prepared from the cells according to the method described in Materials and Methods. The cytosol and total membrane fractions were prepared by centrifugation at 367,000 *g* for 30 min as the supernatant and the pellet, respectively. A: The membrane fraction (60 μg of protein) was sonicated and analyzed by ultracentrifugation as described in Materials and Methods. The samples were separated into a pellet fraction (fraction 1) and five fractions (fractions 2–6 from the bottom to the top). Each fraction was subjected to SDS-PAGE and analyzed by Western blotting using a rabbit anti-caveolin-1 antibody (gel at bottom). Lipid was extracted from each membrane fraction and analyzed by TLC to determine radioactivity in DG. B and C: The cytosol fraction (350 μg protein/7 ml) was overlaid on top of the sucrose solutions at a density of 1.17 g/ml (18 ml) and centrifuged at 49,000 rpm for 48 h. The solution in the centrifuge tube was collected from the bottom into 12 fractions, and lipids were extracted. Radioactivities of phosphatidylcholine (PC; circles) and cholesterol (CH; triangles) (B) and of DG (C) were determined after the lipid was separated by TLC.

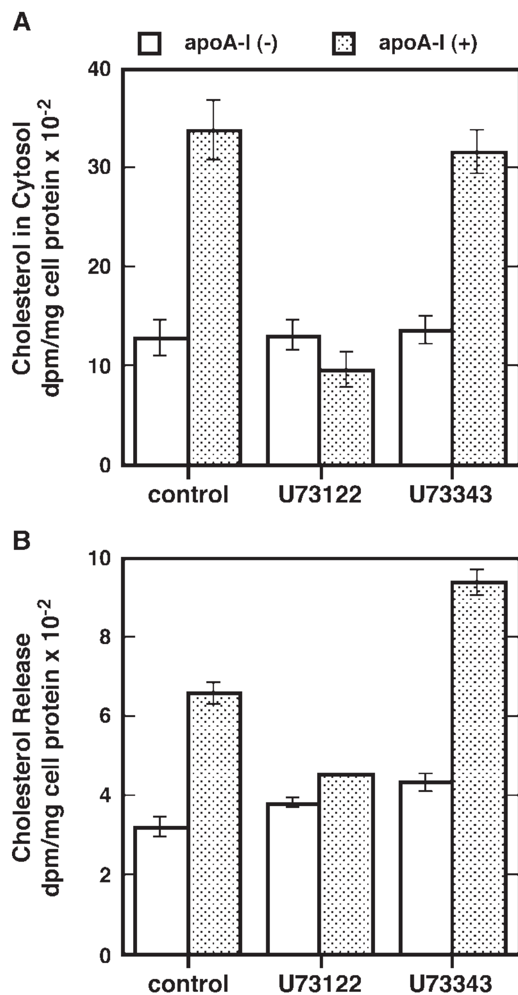


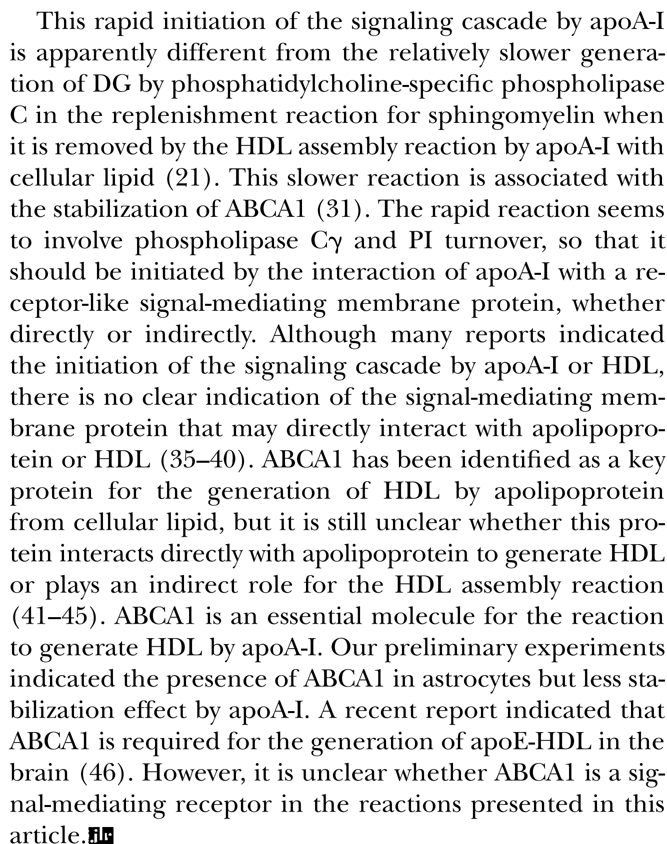

**Fig. 7.** Effects of U73122 on cholesterol trafficking in mouse astrocytes. A: The cells were pulse-labeled for 3 h with 20 μCi of [$^3$H]acetate in 1 ml of 0.02% BSA/DMEM followed by washing and medium replacement with fresh 0.02% BSA/DMEM containing 1 mM sodium acetate. The cells were treated with (dotted columns) or without (open columns) 5 μg/ml apoA-I in the presence or absence of U73122 (10 μM) or U73343 (10 μM) for 90 min. After washing, the cytosol was prepared and lipid was extracted. Radioactivity of cholesterol was determined after separation of lipid by TLC. B: The cells were labeled for 16 h with 20 μCi/ml [$^3$H]acetate in 0.02% BSA/DMEM, and the medium was replaced with fresh 0.02% BSA/DMEM containing 1 mM sodium acetate. The cells were incubated with (dotted columns) or without (open columns) 5 μg/ml apoA-I in the presence or absence of U73122 (10 μM) or U73343 (10 μM) for 4 h. Lipids were extracted from the conditioned medium, and radioactivity in cholesterol was determined.

cannot completely exclude the possibility of the participation of phospholipase Cβ in DG production.

The increase of DG production by apoA-I was accompanied by the translocation of protein kinase Cα to the cytosol in the astrocytes of rat, mouse, and apoE-KO mouse. Thus, the reactions seem to be independent of the influence of endogenously synthesized apoE in astrocytes. The increase of protein kinase Cα in the cytosol was again exclusively in the CLPP fraction. U73122 inhibited the translocation of protein kinase Cα to CLPP, so that it is reasonable to assume that this translocation occurs downstream

of DG production as a signal initiated by apoA-I. This view is consistent with our previous findings that the differentiated rat vascular smooth muscle cells that produce cholesterol-poor HDL by apolipoproteins generate cholesterol-rich HDL after stimulation of protein kinase C by phorbol ester and that protein kinase C inhibitors decreased the apoA-I-mediated cholesterol release in macrophages (34). Further investigation is required to clarify whether the translocation of these signal-related molecules takes place to the same lipid-protein particle or to different particles that happen to have the same density.

In agreement with our previous finding that apoA-I induces the translocation of caveolin-1 and newly synthesized cholesterol to the CLPP fraction, this fraction may play a role in intracellular cholesterol transport to the plasma membrane when HDL is generated by apoA-I and may also provide a site for the initiation of signal transduction to induce such cholesterol trafficking. Interestingly, protein kinase Cα phosphorylated at serine-657 was mainly recovered from the free protein fraction in cytosol, although it is increased in the CLPP fraction also by apoA-I stimulation (28). This finding indicates the possibility that the enzyme is translocated to the CLPP and dissociated from the particle by serine phosphorylation. There is no further information for the reactions after the activation of protein kinase Cα.



This rapid initiation of the signaling cascade by apoA-I is apparently different from the relatively slower generation of DG by phosphatidylcholine-specific phospholipase C in the replenishment reaction for sphingomyelin when it is removed by the HDL assembly reaction by apoA-I with cellular lipid (21). This slower reaction is associated with the stabilization of ABCA1 (31). The rapid reaction seems to involve phospholipase Cγ and PI turnover, so that it should be initiated by the interaction of apoA-I with a receptor-like signal-mediating membrane protein, whether directly or indirectly. Although many reports indicated the initiation of the signaling cascade by apoA-I or HDL, there is no clear indication of the signal-mediating membrane protein that may directly interact with apolipoprotein or HDL (35–40). ABCA1 has been identified as a key protein for the generation of HDL by apolipoprotein from cellular lipid, but it is still unclear whether this protein interacts directly with apolipoprotein to generate HDL or plays an indirect role for the HDL assembly reaction (41–45). ABCA1 is an essential molecule for the reaction to generate HDL by apoA-I. Our preliminary experiments indicated the presence of ABCA1 in astrocytes but less stabilization effect by apoA-I. A recent report indicated that ABCA1 is required for the generation of apoE-HDL in the brain (46). However, it is unclear whether ABCA1 is a signal-mediating receptor in the reactions presented in this article.

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