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Short communication

Carbachol-induced Phosphorylation of Phospholipase D1 through Protein Kinase C is required for the Activation in COS-7 cells

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Phospholiapse D (PLD), and phosphatidic acid generated by it, have been implicated in receptor-mediated intracellular signaling. Carbachol (CCh) is known to activate PLD1, and protein kinase C (PKC) is known to mediate in this signaling pathway. In recent reports (Kim *et al.*, 1999b; Kim *et al.*, 2000), we published our observations of the direct phosphorylation of PLD1 by PKC and we described the phosphorylation-dependent regulation of PLD1 activity. In this study, we investigated the phosphorylation and compartmentalization of PLD1 in terms of CCh signaling in M3 muscarinic receptor (M3R)-expressing COS-7 cells. CCh treatment of COS-7 cells transiently co-expressing PLD1 and M3R stimulated PLD1 activity and induced direct phosphorylation of PLD1 by PKC. The CCh-induced activation and phosphorylation of PLD1 was completely blocked upon pretreatment of the cells with PKC-specific inhibitors. We looked at the localization of the PLD1 phosphorylation by PKC and found that PLD1 was mainly located in the caveolin-enriched membrane (CEM) fraction. Based on these results, we conclude that CCh induces the activation and phosphorylation of PLD1 via PKC and that the phosphorylation of PLD1 occurs in caveolae.

Keywords: Carbachol, Caveolae, M3 muscarinic receptor, Phospholipase D1, Protein kinase C.

Introduction

A variety of ligands activate phospholipase D (PLD) in many cell lines. PLD hydrolyzes phosphatidylcholine generating phosphatidic acid (PA) and choline. As a second messenger, PA can stimulate various intracellular signalings that are involved in multiple physiological processes. These include respiratory bursts, secretion, vesicle trafficking, mitosis, and meiosis. (English, 1996; Olson and Lambeth, 1996; Exton, 1998)

One of mamalian PLD isozymes, PLD1, is known to be regulated by protein kinase C (PKC) *in vivo* and *in vitro*. In previous reports, we suggested a regulatory mechanism of PLD1 by PKC. PKCα was found to be associated with PLD1 after phorbol 12-myristate 13-acetate (PMA) stimulation (Lee *et al.*, 1997). Furthermore, phosphorylations of multiple residues of PLD1 occurred in cells upon PMA-stimulation. Serine 2, threonine 147, and serine 561 of PLD1 were determined as sites directly phosphorylated by PKC (Kim *et al.*, 1999b). Mutations at these phosphorylation sites revealed that the phosphorylation of these sites was required for the activation of PLD1 transiently expressed in COS-7 cells (Kim *et al.*, 2000).

A variety of heterotrimeric G protein coupled receptors (GPCRs) in a wide range of cell types has been reported to mediate PLD stimulation in response to specific agonists. GPCR activates phospholipase C (PLC) β isozymes by either GTP-activated α subunits or free βγ dimers (Rhee and Choi, 1992; Sternweis, 1994). The activated PLCβ then hydrolyzes phosphatidylinositol 4, 5-bisphosphate to generate inositol 1,4,5-triphosphate and 1,2-diacylglycerol, which elevates the intracellular Ca^{2+} concentration and PKC activity, respectively (Singer *et al.*, 1997; Exton, 1999). Five subtypes of the muscarinic acetylcholine receptor (mAChR) (M1-M5) have been identified and their unique genes cloned (Bonner *et al.*, 1987, 1988). Carbachol (CCh) has been known to cause PLD activation in human embryonic kidney cells expressing the human M3 mAChR (Sandmann *et al.*, 1991; Schmidt *et al.*, 1995; Zhang *et al.*, 1999). PLD can be stimulated by various mechanisms apparently involved in PKC activity and calcium-dependent events, as well as tyrosine phosphorylation (BoyanoAdanez *et al.*, 1997; Zhang *et al.*, 1999; Min *et al.*, 2000).

Caveolae are small flask-shaped invaginations of the

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plasma membrane that are characterized by high levels of cholesterol and glycosphingolipids as well as scaffolding proteins, the principals of which are the caveolins, 20-24 KDa integral membrane proteins that undergo homooligomerization (Sargiacomo *et al.*, 1993; Monier *et al.*, 1995). These specialized lipid microdomains play a role in the compartmentalization of a number of plasma membrane-linked signal transduction pathways, including those mediated by the receptor tyrosine kinase. Muscarinic acetylcholine receptors are targeted to plasmalemnal caveolae in adult rat ventricular myocytes upon stimulation (Feron *et al.*, 1997). In addition, a number of GPCRs including the B2 bradykinin, β-adrenergic, cholecystokinin, endothelin, angiotensin II and calcium-sensing receptors have been shown to be located within caveolae under native conditions or upon agonist stimulation (Anderson, 1998; Okamoto *et al.*, 1998).

Here, we report the results of our study of the regulation mechanisms of PLD1 activation and its phosphorylation by PKC upon stimulation with CCh. PKC is involved in the activation of PLD1 upon CCh stimulation, which occurs concomitant with the phosphorylation of PLD1. Furthermore, we show evidence of the subcellular compartmentalization of PLD1 phosphorylation, specifically in the caveolae.

Materials and Methods

Materials The [^{3}H]myristic acid and the chemiluminescence kit (ECL system) were purchased from Amersham International (Buckinghamshire, United Kingdom). Dulbecco's modified Eagle's medium (DMEM) and the LipofectAMINE Reagent were from GIBCO-BRL (Grand Island, USA). Fetal calf serum (FCS) was obtained from PAA Laboratories, Inc. (Parker Ford, USA). GF 109203X and Ro-31-8220 were from Calbiochem-Novabiochem Co. (La Jolla, USA). Phenylmethylsulfonylfluoride, leupeptin, and aprotinin were obtained from Roche Molecular Biochemicals. Silica gel 60 thin-layer chromatography plates were from MERCK (Darmstadt, Germany). Immobilized Protein A was purchased from PIERCE (Rockford, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG, IgM, and IgA, came from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, USA). An antibody against the C-terminal region of PLD1 and phospho-PLD1 were made and purified as described previously (Kim *et al.* 2000). The anti-caveolin-1 polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, USA).

Cell culture Monolayer cultures of COS-7 cells were maintained in 150 mm-diameter tissue culture dishes in a growth medium composed of DMEM supplemented with 10% (v/v) FCS. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

PLD expression in COS-7 cells Transfection was performed with LipofectAMINE according to the manufacturer's instructions. COS-7 cells were seeded into 6 wells at 2×10^5 cells/well and grown for 24 hr. One μg of DNA and 6 μl of LipofecAMINE were separately diluted into 100 μl serum free DMEM, mixed, and incubated at room temperature for 15 min. The cells were washed twice with serum free DMEM and overlaid with 1 ml of serum free DMEM to which the DNA-lipid complexes were then added. The cells were incubated for 5 h at 37°C in a humidified atmosphere of 5% CO_2, 95% air, after which the medium was replaced with DMEM supplemented with 10% (v/v) FCS.

Phospholipase D assay Cells were serum-starved in a serum-free medium for 24 h, after which they were loaded with 3 μCi/ml [^{3}H]myristic acid for 4 h and then treated with CCh in the presence of 0.4% (v/v) butanol. Phosphatidylbutanol (PBtOH) formation was measured as described before with a slight modification (Lee *et al.*, 2000). After incubation with CCh, the medium was removed, and 0.4 ml of methanol was added to each well. The cell debris in each well was then scraped into an Eppendorf tube, and 0.4 ml of chloroform and 0.1 N HCl were added. After vortexing, the tubes were centrifuged at 15,000 × g for 1 min, and the organic phase was harvested, concentrated, and spotted onto a Silica Gel 60 thin layer chromatography plate, which was then developed with chroloform : methanol : acetic acid (110 : 10 : 10 by volume). A Fuji BAS-2000 image analyzer (FUJI FILM CO., LTD.) was used to determine the amount of labeled PBtOH and total lipids.

Immonoprecipitation and immunoplot analysis of PLD1 Immunoprecipitation and immunoblot analysis were done as described previously (Kim *et al.*, 2000). Briefly, the cells were lysed in 1 ml of lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.5 mM EGTA, and 10 mM NaCl, 1% Triton X-100, and 1% sodium cholate) containing protease inhibitors (0.5 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, and 5 μg/ml aprotinin) and phosphatase inhibitors (30 mM NaF, 1 mM Na_3VO_4, and 30 mM $Na_4O_7P_2$). After centrifugation (12,000 × g for 15 min), equal amounts of soluble extract were incubated with 2 μg of anti-C-terminal PLD1 antibody and 25 μl of immobilized protein A resin. The immunoprecipitated proteins were then separated in 6-16% gradient SDS-polyacrylamide gels. The culture supernatant of a hybridoma cell line secreting anti-phospho-PLD1, or 0.4 μg/ml anti-C-terminal PLD1 antibody, and anti-caveolin-1 polyclonal antibody (1 : 1,000) were used as primary antibodies.

Isolation of caveolin-enriched membrane Cavolin-enriched membrane fractions were prepared as previously described (Kim *et al.*, 2000) with some modification. The COS-7 cells were treated with CCh for 1 min and then washed with phosphate-buffered saline and scraped into 2 ml of 500 mM sodium carbonate pH 11.0. The cell suspension was then homogenized using a Dounce homogenizer and a Polytron tissue grinder and lyzed by sonication. The homogenate was adjusted to 40% sucrose by addition of 80% sucrose prepared in a MBS buffer (25 mM MES-NaOH pH 6.5, 150 mM NaCl) and placed in the bottom of a centrifuge tube. Four ml of 30% and then 4 ml of 5% sucrose in a MBS buffer were layered on top. The sample was centrifuged at 100,000 × g for 6 h. One ml fractions were collected from the top, yielding a total of 12 fractions.

Results

CCh-induced activation and phosphorylation of PLD1 We reported on the PKC-dependent regulatory mechanisms of

PLD1 (Lee *et al.*, 1997; Kim *et al.*, 1999b, 2000). To further investigate the molecular mechanism of PLD1 activation by CCh, we transiently co-transfected COS-7 cells with rat PLD1b (rPLD1b) and the m3 muscarinic receptor (M3R). PLD activity was measured as the accumulation of PBtOH, which is a stable metabolite formed by PLD in the presence of a primary butanol. To define the dose-dependent PLD1 activation by CCh, we measured PLD activity for 15 min after the addition of various doses of CCh. PLD1 activity was increased at the micromolar concentration of CCh, and the maximal response was obtained with 100 μM CCh (Fig. 1A). The time-dependence of PLD1 activity was measured at the 100 μM CCh over various time intervals. The accumulation of PBtOH was increased up to 20 min (Fig. 1B). Recently, we reported multiple phosphorylation sites on PLD1, serine 2, threonine 147, and serine 561, discovered upon treatment of PMA (Kim *et al.*, 1999b, 2000). This finding suggested the involvement of PKC in the phosphorylation of PLD1. We developed a monoclonal antibody that specifically recognized the threonine 147 phosphorylation site on PLD1 (anti-phospho-PLD1 antibody) (Kim *et al.*, 2000) and were thus able to easily monitor phosphorylation of PLD1. The CCh-induced PLD1 phosphorylation that occurred was maximally detected at a 10 μM concentration (Fig. 2A). PLD1 phosphorylation showed a peak after 1 min and sustained phosphorylation levels up to 20 min (Fig. 2B). These results, therefore confimed the activation and phosphorylation of PLD1 after CCh stimulation.

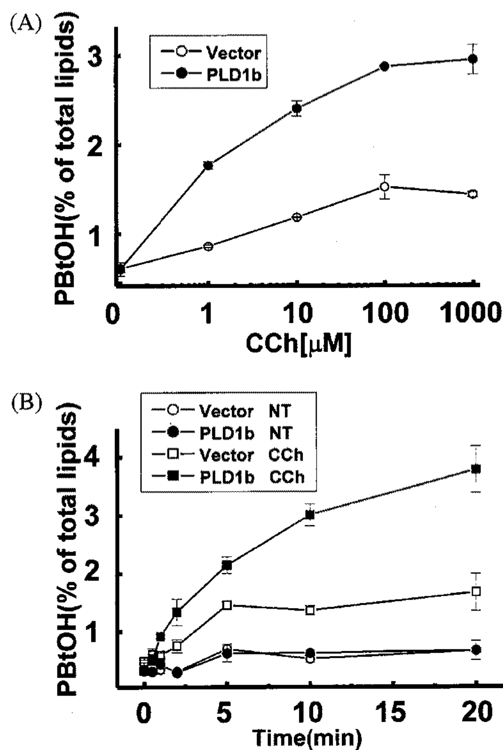

Fig. 1. Concentration and time-dependent activation of PLD1 by CCh in COS-7 cells. 2×10^5 COS-7 cells were co-transfected with the M3R and rPLD1b (or vector DNA), respectively. 0.5 μg DNA was used. 24 h after transfection, the cells were starved for 24 h prior to incubation with 4 μCi/ml [^{3}H]myristic acid for 3 h and then treated with various doses of CCh (A) for 15 min and with 100 μM CCh for various lengths of time (B) in the presence of 0.4% (v/v) 1-butanol. Analysis of [^{3}H]PBtOH was performed as described in "Materials and Methods." The amount of [^{3}H]PBtOH is expressed as percentage of total radioactivity recovered from the TLC plate. Data shown are means S.E.M. from two different experiments, each performed in duplicate.

Phosphorylation of PLD1 is required for the activation of PLD1 upon treatment with CCh GPCRs activate PLCβ isozymes, which results in an increase in intracellular Ca^{2+} concentration and the activation of PKCs. It has been known that CCh stimulation of M3Rs results in the activation and translocation of PKC (Rumenapp *et al.*, 1995). Recently, we reported the PKC-mediated PLD1 phosphorylation by treatment of PMA in COS-7 cells. To investigate the involvement of PKC in the activation and phosphorylation of PLD1 by CCh, we used the specific PKC inhibitors, GF109203X and Ro-31-8220. As shown in Fig. 3, pretreatment of COS-7 cells that had been co-transfected with rPLD1 and M3R with the PKC inhibitors, GF 109203X and Ro-31-8220, resulted in the complete inhibition of the CCh-

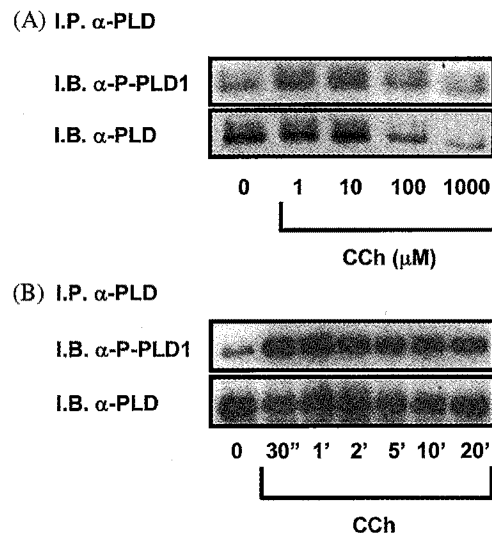

Fig. 2. Concentration and time-dependent phosphorylation of PLD1 by CCh in COS-7 cells. A. COS-7 cells co-transfected with M3 muscarinic receptor and rPLD1b were treated with various concentrations of CCh (A) for 1 min and with 100 μM CCh for various lengths of time (B). The cells were lysed with 1 ml of lysis buffer containing protease inhibitors and phosphatase inhibitors. After centrifugation (12,000 × g for 15 min), equal amounts of soluble extract were incubated with 2 μg of anti-C-terminal PLD1 antibody and 25 μl of immobilized protein A resin. The immunoprecipitated proteins were then separated in a 6-18% gradient SDS-polyacrylamide gel. The culture supernatant of a hybridoma cell line secreting anti-phospho-PLD1 (P-PLD1) or 0.4 μg/ml anti-C-terminal PLD1 (PLD1) antibody were used as primary antibodies.

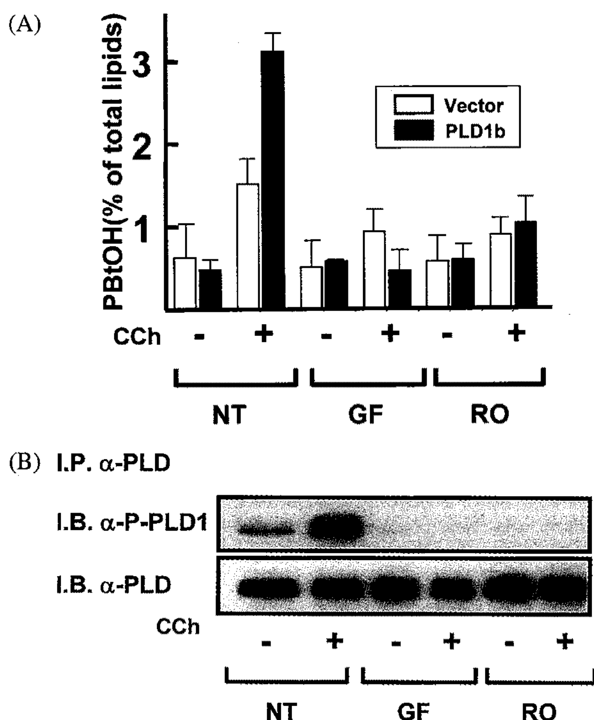

Fig. 3. Inhibition of CCh-induced PLD1 activation and phosphorylation by treatment of PKC inhibitors. (A) COS-7 cells were preincubated with 5 μM GF 109203X and 5 μM Ro-31-8220 for 15 min and then stimulated with 100 μM CCh for 15 min in the presence of 0.4% (v/v) 1-butanol. Analysis of [^{3}H]PBtOH was performed as described in "Materials and Methods." The amount of [^{3}H]PBtOH is expressed as percentage of total radioactivity recovered from the TLC plate. Data shown are means±S.E.M. from two separate experiments, each performed in duplicate. (B) COS-7 cells were preincubated with 5 μM GF 109203X and 5 μM Ro-31-8220 for 15 min, then treated with 100 μM CCh for 1 min, and finally the cells were lysed with 1 ml of lysis buffer containing protease inhibitors and phosphatase inhibitors. The immunoprecipitated proteins were then separated in a 6-18% gradient SDS-polyacrylamide gel. The culture supernatant of a hybridoma cell line secreting anti-P-PLD or 0.4 μg/ml anti-PLD1 antibody were used as primary antibodies.

induced PLD1 activation and phosphorylation. From these results, we deduce that phosphorylation may be involved in the CCh-induced PLD1 activation via PKC.

PLD1 phosphorylation in CEM upon treatment of CCh It is known that PLD1 exists in multiple subcellular locations in the plasma membrane and vesicle structures. Previously, we reported that PLD1 was localized in the plasma membrane, in particular in the CEM fraction, to which PKCα translocated after stimulation of PMA (Kim *et al.*, 1999a). We have also shown that the event of the PLD1 phosphorylation occurred in the CEM fraction upon treatment of the cells with PMA (Kim *et al.*, 2000). To define the location of the PLD1 phosphorylation upon CCh treatment, we transiently co-

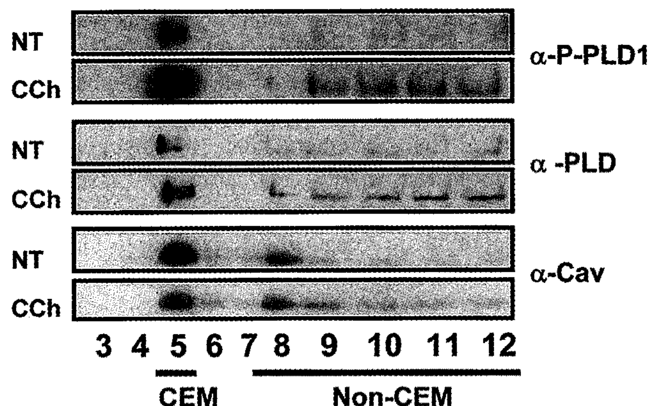

Fig. 4. CCh-induced PLD1 phosphorylation in the CEM fraction. COS-7 cells were incubated in the absence or presence of 100 μM CCh for 1 min. CEMs were prepared by a sucrose density gradient centrifugation as described in "Materials and Methods." 25 μl of each fraction was subjected to 6-18% gradient SDS-PAGE followed by immunoblot analysis with specific antibodies directed against PLD1, P-PLD1, and caveolin-1 (Cav).

transfected COS-7 cells with rPLD1 and M3R, and we resolved the CEM fraction checking for the location of the PLD1 phosphorylation. Caveolin was enriched in the CEM fraction indicating a good preparation. As shown in our previous reports, PLD1 was highly enriched in the CEM fraction that was independent of CCh treatment. Although PLD1 was located in both the CEM and non-CEM fractions, the phosphorylation of PLD1 mainly occurred in the CEM fraction (Fig. 4).

Discussion

PKC is well known as a major activator of PLD1 in various cell lines. From our previous report, several regulatory mechanisms of PLD1 activation through PKC have been suggested. PLD1 and PKCα are co-localized in the CEM fraction and form a direct complex upon treatmnet with PMA in several cell lines (Lee *et al.*, 1997; Kim *et al.*, 1999a; Kim *et al.*, 2000). Furthermore, we identified several sites of phosphorylation on PLD1 activation in PMA-stimulated cells and showed that mutations of these sites abolish the PMA-induced PLD1 activation (Kim *et al.*, 1999b, 2000). However, it remained to be elucidated whether or not PKC activated by GPCR was linked to the phosphorylation of PLD1 and its location.

The pattern of the PLD1 phosphorylation induced by PMA is complex. We identified more than 9 phosphopeptides in two-dimensional phosphopeptide mapping (Kim *et al.*, 1999b). However, only 4 phosphopeptides were generated by PKC directly by the phosphorylation of serine 2, threonine 147, serine 561, plus an unknown site. Furthermore, mutants in which these phosphorylation sites were substituted with alanine showed significant reduced PMA-induced PLD1

activity (Kim *et al.*, 2000). In order to investigate the CCh-induced PLD1 phosphorylation, we monitored the phosphorylation of PLD1 by use of an anti-phospho-PLD1 antibody specifically recognizing the phosphorylation of threonine 147. The phosphorylation at threonine 147 correlates with PLD1 activity (Fig. 1 and 2). The CCh-induced PLD1 activity continuously increased up to 20 min and the phosphorylation remained sustained up to this time. Furthermore, PKC inhibitors attenuated both activation and phosphorylation of PLD1 (Fig. 3). These data suggest that the phosphorylation of PLD1 through PKC is required for PLD1 activation.

PLD activity and localization has been reported in such diverse subcellular organelles as plasma membrane, Golgi vesicles, endoplasmic reticulum, secretory vesicles, and the nuclear membrane (Balboa *et al.*, 1995; Ktistakis *et al.*, 1995; Provost *et al.*, 1996; Whatmore *et al.*, 1996; Banno *et al.*, 1997; Baldassare *et al.*, 1997). Recently, we reported that PLD1 was localized in plasma membrane, especially in the caveolae (Kim *et al.*, 1999c; Kim *et al.*, 1999a), and that the phosphorylation of PLD1 occurred only at these sites (Kim *et al.*, 2000). CCh also induced the phosphorylation of PLD1 in the CEM fraction in COS-7 cells (Fig. 4). Caveolae have been implicated in exocytosis and endocytic pathways, such as transcytosis and pinocytosis (Schnitzer *et al.*, 1994; Scheiffele *et al.*, 1998). PKCa and RhoA, which are known as the activators of PLD1, have been found in caveolae and implicated in the formation of caveolae structures (Senda *et al.*, 1997; Gingras *et al.*, 1998). Previously, it was reported that M2 mAchR was targeted, upon agonist stimulation, to caveolae and sequestrated through the caveolae pathway, finally resulting in a receptor desensitization and resensitization process, or both (Feron *et al.*, 1997; Dessy *et al.*, 2000). Although, it is still uncertain if PLD1 is involved in the caveolae-mediated exo- and endocytosis, it is possible that increased PA levels, generated as a consequence of PLD activity, could function in these processes.

In conclusion, we showed the activation and phosphorylation of PLD1 upon the stimulation of CCh and the phosphorylation of PLD1 in caveolae.

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