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Activation of protein kinase C modulates BACE1-mediated β -secretase activity

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Abstract

β -site APP cleaving enzyme 1 (BACE1) is the β -secretase responsible for generating amyloid- β (A β) peptides in Alzheimer's disease (AD). Previous studies suggest that activation of protein kinase C (PKC) modulates the β -secretase-mediated cleavage of APP and reduces the production of A β . The mechanism of PKC-mediated modulation of β -secretase activity, however, remains elusive. We report here that activation of PKC modulated β -secretase activity through either suppressing the accumulation or promoting the translocation of BACE1 protein in a cell type-dependent manner. We found that activation of PKC suppressed the accumulation of BACE1 protein in fibroblasts through an enhancement of intracellular protease activities. In neurons, activation of PKC did not alter the expression level of BACE1, but led to more BACE1 translocated to the cell surface, resulting in a decreased cleavage of APP at the β 1 site. Together, Our findings provide novel mechanisms of PKC-mediated modulation of β -secretase activity, suggesting that alteration of the intracellular trafficking of BACE1 may serve as a useful therapeutic strategy to lower the production of A β in AD.

Keywords

BACE1; APP; PKC; protein degradation; protein translocation; amyloid β ; β -secretase; fibroblast; neuron

1. Introduction

A wide array of studies have demonstrated that genetic mutations linked to Alzheimer's disease (AD) invariably increase the production and deposition of amyloid β . (A β) peptides, strongly supporting the idea that excessive accumulation of A β peptides contributes to the pathogenesis of AD (10). A β peptides are derived from amyloid precursor protein (APP) by sequential endoproteolytic cleavages of β - and γ -secretases (25). Alternatively, APP is cleaved by α - and γ -secretases to generate the non-pathogenic p3 fragments (25). β -APP cleaving enzyme 1 (BACE1) is the β -secretase responsible for generation of A β peptides (28). BACE1 cleaves APP at both β 1 and β 11 sites and generates β 1 and β 11 carboxyl terminal fragments (CTF) of APP (4;28). Genetic deletion of BACE1 abolishes the generation of APP β 1 and β 11 CTFs, prevents the generation and deposition of A β peptides, and rescues cognitive impairments and

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other neuropathological abnormalities, such as dystrophic neurites, astrogliosis, and microgliosis in transgenic mice overexpressing mutant APP (14;20). Although cognitive deficits, emotional alterations, and premature lethality are reported in BACE1-deficient mice (11;14;20), it seems that partial inhibition of BACE1 expression, which does not cause obvious side effects, is sufficient to reduce amyloid accumulation and improve the cognitive function of APP transgenic mice (14;27). Thus, we decided to investigate the intracellular signaling transduction pathways that partially suppress the expression and function of BACE1 for their potential therapeutic values in AD.

Previous studies suggest that activation of protein kinase C (PKC)-mediated intracellular signal transduction pathways affects α and β -secretase-mediated cleavage of APP, resulting in a reduction of A β peptides (1;12;24). However, the mechanism of PKC-mediated inhibition of β -secretase activity is unknown. To address this question, we examined the role of PKC in BACE1 protein expression and its β -secretase activity in mouse fibroblasts and primary cultured cortical neurons. We found that the level of BACE1 protein in mouse fibroblasts was down regulated by phorbol myristate acetate (PMA), a PKC activator, through an enhancement of intracellular protease-mediated protein degradation pathways. The expression of BACE1 protein in primary cultured mouse cortical neurons, however, was not affected by PMA treatment. Instead, application of PMA increased the presentation of BACE1 as well as APP proteins at the plasma membrane, resulting in a reduction of APP β 1 CTF in neurons. Therefore, that the PMA-induced increase of BACE1 degradation in fibroblasts and BACE1 translocation to the cell surface in neurons underlines novel mechanisms of PKC-mediated suppression of β -secretase activity at APP β 1 site.

2. Materials and methods

2.1 Generation of mouse fibroblasts

Fibroblast cultures were established from postnatal day 1 wild type and BACE1^{-/-} pups. Briefly, the skins were dissected, minced, plated on culture dishes and incubated at 37°C in medium containing Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) and 10% FBS (Invitrogen) for 7 d. The resulting monolayer of primary fibroblasts were dissociated by trypsin digestion and expanded, and then immortalized with transfection of large T antigen.

2.2 Cell culture and treatment

Fibroblast cell lines were cultured in DMEM (Invitrogen) supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μ g/ml) (Invitrogen) and maintained at 37°C in a humidified 5% CO₂ atmosphere. The cells (10⁶) were seeded in 6 well plates and stimulated 18 hr later with PMA (Sigma, St. Louis, MO) for various times. Where indicated, the cells were preincubated with appropriate inhibitors 30 min before PMA stimulations and the inhibitors were maintained during the stimulations. PMA and all inhibitors, except for chloroquine (Sigma), were solubilized in Dimethyl sulfoxide (DMSO). Chloroquine was solubilized in water.

2.3 Western blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 1% nonidet P-40 (NP-40), 0.4% sodium dodecyl sulphate (SDS) and a protease inhibitor mixture (Roche Diagnostic, Alameda, CA). Whole cell lysates were prepared from the supernatant fraction after centrifugation at 12,000 $\times$ g for 10 min at 4°C. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumin (BSA) as standard. For Western blotting, 10 μ g of total protein were separated by NuPage 4–12% BisTris-polyacrylamide gel electrophoresis (Invitrogen)

using MES running buffer (Invitrogen) for BACE1 and full-length APP or by Novex 16% Tricine gel electrophoresis (Invitrogen) for APP CTFs. Separated proteins were then transferred to polyvinylidene difluoride membranes (PVDF) and incubated with antibody specific for BACE1 (4), or the C-terminal of APP (Sigma) at a 1:2000 dilution. Bound antibodies were detected by the enhanced chemiluminescent method. Membranes were stripped to prepare them for a second round of probing with β -actin or β -tubulin antibodies (Chemicon, Temecula, CA; 1:5000 dilution).

2.4 Surface biotinylation

Fibroblasts and neurons were grown in 35-mm² dishes and treated with vehicle (DMSO) or PMA for the indicated times. Cell surface biotinylation was performed as described previously (19). Briefly, cells were cooled on ice, washed two times with ice-cold labeling buffer containing: 125 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, 1 mM NaH₂PO₄, 10 mM dextrose, 2.5 mM CaCl₂, 1.25 mM MgCl₂, and 5% CO₂, and then incubated with labeling buffer containing 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce) for 20 min on ice. Unreacted biotinylation reagent was washed with ice-cold labeling buffer and quenched by two successive 20 min washes in labeling buffer containing 100 mM glycine on ice, followed by two washes in ice-cold TBS (50 mM Tris, pH 7.5, 150 mM NaCl). Cultures were harvested in modified RIPA buffer (1% Triton X-100, 0.5% SDS, 0.5% deoxycholic acid, 50 mM NaPO₄, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and protease inhibitor complex). The lysates were cleared by centrifugation for 15 min at 14,000× g at 4°C. The resulting supernatant was incubated with 100 μ l of 50% NeutraAvidin agarose (Pierce) for 3 hr at 4°C. The NeutraAvidin agarose was washed five times with RIPA buffer. Bound proteins were eluted with SDS sample buffer by boiling for 15 min.

2.5 β -Secretase activity assay

The quantification of β -secretase activity in fibroblast cell lines or primary cultured neurons was carried out according to manufacturer's instructions with minor modifications (R&D Systems, Minneapolis, MN). Briefly, fibroblast cells or neurons were washed in ice-cold PBS and incubated in extraction buffer for 1 hr on ice. Cells were homogenized in extraction buffer, and centrifuged at 10,000 × g for 1 min. Supernatant (50 μ l) was added to each well in microplate and mixed with 50 μ l 2 × reaction buffer and 5 μ l substrate. The plates were incubated in the dark at 37°C for 1.5 hr and then read on a fluorescent microplate reader.

2.6 RNA extraction and reverse transcriptase-PCR

Total RNA was extracted using TRIZOL reagent according to the manufacturer's instructions (Invitrogen). Reverse transcriptase (RT)-PCR was performed using SuperScript™ III one-step RT-PCR System (Invitrogen). The following primers were used: for BACE1, 5'-GTCATACTCAGGCTACCCGGC-3' and 5'-TCTTCTGCTGACTTTGGCCAG-3'; and for β -actin, 5'-TGTGATGGTGGGAATGGGTCAG-3' and 5'-TTTGATGTCACGCACGATTTC-3'. RT-PCR conditions were performed as follows: 1 cycle of 50°C for 30 min for cDNA synthesis, 1 cycle of 94°C for 2 min for pre-denaturation, 26 cycle (for BACE1) or 21 cycle (for β -actin) for DNA amplification (denature at 94°C for 30 s, annealing at 60°C [for BACE1] or 62°C [for β -actin] for 30 s, extension at 72°C for 45 s), and final extension at 72°C for 10 min. PCR products were separated by electrophoresis on 2% agarose gel containing ethidium bromide.

2.7 BACE1 expression construct, mutagenesis, and transfection

Full-length wild type human BACE1 cDNA (4) in pRK5 vector was digested with restriction enzymes *EcoRI/HindIII*. The *EcoRI/HindIII* BACE1 cDNA fragment, which represents the entire coding sequence for wild type human BACE1, was subcloned into pBluescript vector

for mutagenesis. Using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), BACE1 serine residue at amino acid 498 was mutated to alanine (S498A) using the following mutagenesis primers: forward 5'-TTGCTGATGACATCGCCCTGCTGAAGTGA-3' and reverse 5'-TCACTTCAGCAGGGCGATG TCATCAGCAA-3' and following manufacturer's instructions. The resulting BACE1-S498A construct was digested with *EcoRI/HindIII* and cloned back into the pRK5 vector. All constructs were sequenced and verified. Fibroblast cells were transfected with BACE1 cDNAs using Fugene-6 transfection reagent (Roche) according to the supplier's instructions.

2.8 Primary culture of mouse cortical neurons

As described previously (3), newborn (P0) pups were decapitated and the cortices were rapidly removed and placed in 35-mm² petri dishes containing sterile Hanks buffer supplemented with 2.5 mM HEPES, pH 7.4, 35 mM glucose, 1 mM CaCl₂, 1 mM MgSO₄, 4 mM NaHCO₃. The meninges surrounding the tissues were removed and the neurons were dissociated by papain (Worthington, Lakewood, NJ) digestion. Neurons were plated at a density of 10⁶ cells/well in 6-well plate coated with poly-D-lysine (Becton Dickinson, San Jose, CA) in Basal Medium Eagle (BME) (Sigma) supplemented with B27, N2, L-glutamine, and penicillin/streptomycin (Invitrogen). Arabinosylcytosine or β -D-arabinofuranoside cytosine (Sigma) was used to inhibit the growth of glial cells. Cultures were incubated at 37°C with 5% CO₂. 75% of culture medium was changed every 3 days.

2.9 Quantification of DNA and protein and statistics

All the Western blot and DNA gel images were quantified using a Scion Image System (Scion, Frederick, MD). Student *t*-test was used for statistical analysis.

3. Results

3.1 PMA inhibited β -secretase activity and suppressed BACE1 expression in mouse fibroblasts

Although BACE1 protein is enriched in neurons (14), it also expresses in mouse fibroblasts and displays β -secretase activity (Fig. 1A, lane "WT"; and 1B, column "con"). Consistent with previous observations in neurons and other cell lines (14), multiple BACE1 bands were revealed by Western blotting (Fig. 1A), reflecting a series of post-translational modifications of BACE1 protein (9). In addition, several non-specific bands ranging from 50 to 75 kDa were detected by the BACE1 antibody, which also existed in BACE1^{-/-} cell lysates (Fig. 1A, lane "KO").

Activation of PKC has been shown to modulate the activity of α and β -secretase activities and reduce A β production (1;12;24). To determine how activation of PKC modulates BACE1-mediated β -secretase activity, we treated mouse fibroblasts with 10 μ M PMA, a PKC activator for 6 h and collected the cell extracts for an *in vitro* β -secretase activity assay in which a 12 amino acid polypeptides containing the β 1 site of APP was used as the substrate for BACE1 (in Method section). We observed a significant decrease in β -secretase activity in PMA-treated fibroblasts (Fig. 1B, *n* = 4, *p* < 0.0001). To address the potential mechanism of PMA-induced inhibition of β -secretase activity, we examined the expression of BACE1 protein in fibroblasts following PMA treatment. We found that PMA reduced BACE1 protein expression in fibroblasts (Fig. 1C–D). As a control, PDD (4 α -phorbol 12,13-didecanoate), an inactive structural analog of PMA (17), did not alter BACE1 protein expression under the same experimental paradigm (Fig. 1C–D). Moreover, the modulation of BACE1 protein expression by PMA in fibroblasts was both dose- and time-dependent (Fig. 1C–F). In addition, the effects

of PMA were reversible as evidenced by a complete recovery of BACE1 protein expression 24 h after treatment (Fig. 1E–F).

To examine the specificity of PMA-induced effects, we pretreated the fibroblasts with PKC inhibitors, Ro31-8220 (10 μ M) or Rottlerin (5 μ M) for 30 min and then co-incubated with PMA (10 μ M) for 6 h. Ro31-8220 is a specific but broad-spectrum PKC inhibitor and Rottlerin, at the concentration used, is a specific inhibitor of PKC isozyme PKC- δ (8). We found that pretreatment of Ro31-8220 successfully blocked the reduction of BACE1 protein induced by PMA (Fig. 1G–H, $n = 4$, $p < 0.0005$). Whereas pretreatment of Rottlerin had no effect (data not shown), suggesting that PMA-mediated down-regulation of BACE1 protein is PKC dependent, but not through the activation of PKC- δ isoform.

To exclude the possibility that our results were restricted to the particular cell line chosen in this study, we tested three other fibroblast cell lines derived from different mice and found a similar reduction of BACE1 protein following PMA treatment (data not shown).

3.2 The alteration of BACE1 protein expression induced by PMA in fibroblasts was not at the transcriptional level

To determine whether PMA affects the transcription of BACE1, we quantified the messenger RNA (mRNA) level of *BACE1* following PMA treatment by RT-PCR. No significant difference of *BACE1* mRNA level was observed in the presence of 10 μ M PMA for 3 or 6 h (data not shown), indicating that the regulation of BACE1 protein by PMA is most likely post-transcriptional.

3.3 The ERK-related signal transduction pathway is not involved in the PMA-induced suppression of BACE1 protein in fibroblasts

The ERKs (extracellular signal-regulated kinases)-mediated intracellular signaling transduction pathway is one of the major downstream pathways of PKC (6). To evaluate the role of ERKs in PMA-mediated BACE1 suppression, we treated fibroblast cells with an ERK inhibitor, PD98095 (20 μ M) prior to PMA (10 μ M) treatment (Fig. 2). We found that PD98095 did not block the reduction of BACE1 protein induced by PMA treatment, suggesting that PMA-induced effects are likely the ERK pathways independent (Fig. 2A and 2B).

3.4 Phosphorylation of the C-terminal tail of BACE1 was independent of the PMA-induced suppression of BACE1 protein in fibroblasts

Recent studies indicate that phosphorylation of BACE1 at a specific serine residue (S498) of its C-terminal DISLL motif regulates the intracellular trafficking of BACE1 protein (29). To assess the impact of phosphorylation of BACE1-S498 on PMA-induced suppression of BACE1 expression, we replaced this serine residue with alanine to create a non-phosphorylated form of BACE1 protein (BACE1-S498A). Equal concentrations of wild type or mutant BACE1 expression plasmids were transfected into fibroblasts, and treated with PMA 24 h later. We observed a similar rate of BACE1 suppression in wild type and BACE1-S498A transfected cells (Fig. 2C), indicating that the effects of PMA were not dependent on the phosphorylation state of the C-terminal tail of BACE1. In addition, we observed that treatment of PMA induced a molecular mass shift from 70 to 66 kDa of BACE1 in transfected fibroblasts, indicating that activation of PKC may also affect the maturation of exogenous BACE1 protein overexpressed in fibroblasts (Fig. 2C, lanes 6 and 8).

3.5 Lysosome, calpain and cathepsin Inhibitors blocked the PMA-induced degradation of BACE1

Previous studies have suggested that the lysosome and ubiquitin-proteasome pathways are involved in the BACE1 protein degradation (13;22). Consistently, we found that the lysosome pathways primarily mediated the degradation of BACE1 protein in mouse fibroblasts. A substantial elevation of BACE1 expression was observed in cells treated with chloroquine (Ch) for 24 h, which inhibits lysosome hydrolases by reducing the acidification of the endosome/lysosome compartments (Fig. 3A, lane “Ch” and 3B, $n=3$, $p<0.00001$). In contrast, there were no significant changes in BACE1 expression in fibroblasts treated with the proteasome inhibitor, lactacystin (L) for 24 h (Fig. 3A, lane “L” and 3B). MG132 (M), another proteasome inhibitor, was also tested, however, it induced cell death after incubation for 24 h (data not shown).

We then tested whether PMA-induced suppression of BACE1 expression is mediated via lysosome or proteasome-dependant degradation mechanisms. As shown in Fig. 3C, the PMA-induced degradation of BACE1 was effectively blocked in the presence of MG-132 or chloroquine (Fig. 3C, lanes “Mg+P” and “Ch+P”; and 3D). Since the treatment of MG-132 or chloroquine alone for 6 h did not increase BACE1 protein expression in fibroblasts (Fig. 3C, lanes “Mg”, “L”, and “Ch”), it suggested that PMA treatment elevated the activities of these intracellular proteases responsible for BACE1 protein degradation in fibroblasts. However, lactacystin did not exert any effect on BACE1 protein degradation induced by PMA (Fig. 3C, lane “L+P”; 3D). Since lactacystin is a more specific proteasome inhibitor compared to MG-132 (16)], this indicated that proteasome-mediated protein degradation may not play a major role in PMA-induced suppression of BACE1 expression.

Since MG-132 has also been shown to inhibit other proteases, such as calpain, cathepsin and γ -secretase (16), we then tested whether calpain or cathepsin inhibitors affect the expression of BACE1 in the presence of PMA. We found that addition of calpeptin (a calpain inhibitor) or cathepsin inhibitor 1 blocked the degradation of BACE1 induced by PMA (Fig. 3E, lane “Ca+P” and “Ct+P”; and 3F). In addition, treatment with calpain and cathepsin inhibitors alone did not significantly affect BACE1 protein levels in fibroblasts (Fig. 3E, lanes “Ca” and “Ct”; and 3F). Further, we found that BACE1 protein expression in presenilin 1-deficient mouse fibroblasts was also suppressed following PMA treatment, suggesting that γ -secretase activity is not involved in the PMA-mediated degradation of BACE1 (data not shown). Taken together, these results suggest that PMA-mediated degradation of BACE1 in fibroblasts via activation of lysosome and other intracellular proteases, including calpain and cathepsin.

3.6 Treatment with PMA did not affect BACE1 protein expression in primary cultured neurons

Since BACE1 protein is primarily expressed in neurons (14), we then asked if treatment with PMA altered BACE1 protein levels in neurons. Surprisingly, we did not detect any significant alteration of BACE1 protein expression in primary cultured cortical neurons following PMA treatment (Fig. 4A and 4B). In addition, despite previous reports that the degradation of BACE1 protein in neurons is mediated by the proteasome or lysosome pathways (13;22), we did not detect any significant alteration of BACE1 protein expression in primary cultured cortical neurons treated with either MG-132 or chloroquine for 24 h (Fig. 4C and 4D).

3.7 Treatment with PMA increased the translocation of BACE1 protein to the plasma membrane in primary cultured cortical neurons

BACE1 protein is located primarily within intracellular organelles such as the Golgi, secretory vesicles, and endosomes (5;28). In addition, a small percentage of BACE1 protein is also present at the cell surface (31). Since activation of PKC promotes the exocytosis of secretory vesicles and endocytosis of plasma membrane proteins, we examined whether plasma

membrane-bound BACE1 is sensitive to PMA treatment. To test this hypothesis, we quantified surface presentation of BACE1 in neurons following PMA treatment. While BACE1 protein levels in total cell lysates were not altered in PMA-treated neurons (Fig. 5A, middle panel), we found a significant increase in plasma membrane-bound BACE1 protein after PMA treatment (Fig. 5A, upper panel and 5B, $n = 4$, $p < 0.005$), suggesting that more BACE1 protein was translocated onto the plasma membrane following PMA treatment. In contrast, we found that the levels of plasma membrane-bound BACE1 and total BACE1 protein decreased at a similar rate following PMA treatment in fibroblasts (Fig. 5C and 5D, $n = 3$, $p < 0.001$), reflecting a different response to PMA treatment in fibroblasts compared to neurons.

3.8 Treatment with PMA decreased the accumulation of APP β 1 CTF in primary cultured neurons

BACE1 cleaves APP at both the β 1 and β 11 sites to generate APP β 1 and β 11 CTFs (2) (Fig. 6A, middle panel). To address whether translocation of BACE1 to cell surface affects the APP processing by BACE1, we quantified the levels of full-length APP (APP-FL), β and α CTFs in neurons following PMA treatment. Similar to our previous observations on BACE1 (Fig. 5A), treatment of PMA did not affect the level of APP-FL protein (Fig. 6A and 6C), but promoted the translocation of APP to the cell surface (Fig. 6B and 6D, $n = 4$, $p < 0.0001$). The APP C-terminal antibody recognized four major bands ranging from 8–14 kDa that corresponded to APP β 1-phosphorylated (β 1-p), β 11-phosphorylated (β 11-p), β 11/ α -phosphorylated (α -p), and α CTFs (Fig. 6A–B) as previously described (2). Only APP α -p- and α -CTFs were detected in *BACE1*^{-/-} neurons (Fig. 6A–B). Since the accumulation of APP β 1 CTF is extremely low (2) and it is difficult to separate the APP β 11 and α -p CTFs, we only quantified the APP β 1-p, β 11-p and α CTFs in this study. We found that the level of the APP β 1-p CTF was significantly reduced in PMA-treated neurons compared to vehicle-treated ones (Fig. 6A and 6C, $n = 4$, $p < 0.02$). In contrast, the accumulation of the APP β 11-p CTF and α CTF did not alter in whole cell lysates following PMA treatment (Fig. 6A and 6C).

3.9 Treatment with PMA increased the presentation of APP β 11 and α CTFs at the cells surface of primary cultured neurons

Since the levels of both APP and BACE1 were elevated at the cell surface of PMA-treated neurons (Fig. 5A and 6B), we examined whether the levels of APP β CTFs at cell surface were altered. Surprisingly, we could not detect any presentation of APP β 1 CTF at the plasma membrane of either PMA or vehicle-treated neurons (Fig. 6B), suggesting that BACE1 does not cleave APP at the β 1 site of A β at the cell surface. In contrast, both APP β 11 and α CTFs were detected at the cell surface (Figure 6B). Moreover, treatment of PMA increased the presentation of both APP β 11-p and α CTFs at the cell surface (Fig. 6B and 6D, $n = 4$, $p < 0.0001$), correlating with the elevation of both APP and BACE1 at the plasma membrane following PMA treatment.

Discussion

Previous studies have demonstrated that PKC-mediated intracellular signal transduction pathways are involved in modulating APP cleavage at both the α and β sites, resulting in a reduction of A β 1–40/42 generation in cell cultures and mouse brains (1;12;24). However, the mechanism of PKC-mediated regulation of APP cleavage at the β -site is unclear. We report here that there are two independent mechanisms involved with PKC-mediated regulation of β -secretase activity: in fibroblasts, activation of PKC inhibited β -secretase activity by enhancing BACE1 protein degradation; and in neurons, activation of PKC promoted the translocation of BACE1 to the cell surface and decreased the cleavage of APP at the β 1 site.

In fibroblasts, the elevation of lysosome and other intracellular proteases, but not proteasome-mediated protein degradation pathways contributed to PMA-induced reduction of BACE1 protein. Interestingly, pre-incubation of MG-132, a general proteasome inhibitor that also inhibits several other proteases, such as calpain, cathepsin, and γ -secretase, prevented PKC-mediated degradation of BACE1. Calpains, the calcium-dependent cysteine proteases, are involved in the modulation of APP processing (26), and calpain inhibitor increases the generation of both α and β CTF of APP through redistributing APP to the cell surface (23). Although the incubation of calpain inhibitor itself did not affect the accumulation of BACE1, it blocked PMA-induced suppression of BACE1 in fibroblasts. Cathepsin belongs to a family of lysosome-associated proteases. Members of the cathepsin family of proteases have been shown to cleave APP near the secretase-cleaving sites (18). Unlike chloroquine, a general inhibitor of lysosomal activity, cathepsin inhibitor 1 itself did not block the BACE1 degradation in vehicle-treated cells. However, like the calpain inhibitor, cathepsin inhibitor 1 selectively suppressed the PKC-mediated degradation of BACE1 in PMA-treated cells. The precise mechanism of calpain or cathepsin in PKC-mediated suppression of BACE1 accumulation is not clear at this point. A previous study suggests that activation of Calpain is concomitantly with PKC, resulting in a separation of the PKC regulatory domain apart from the catalytic domain, which may enhance the catalytic activity of PKC (15). We speculate that the calpain 1 inhibitor may block the hydrolysis of activated PKC and decrease the activity of the catalytic domain of PKC responsible for the suppression of BACE1 accumulation.

In cultured neurons, activation of PKC did not affect BACE1 expression. Similarly, the treatment with either chloroquine or MG132 did not affect BACE1 levels in neurons, which is inconsistent with previous reports (13;22). The different effects in neurons is likely due to a much higher expression level of BACE1 in neurons compared to non-neuronal cells (14) that could make it difficult to detect minor alterations of BACE1 expression. Alternatively, the degradation of BACE1 protein in neurons may follow a different pathway compared with non-neuronal cells. It will be interesting to address how the expression of BACE1 is differentially regulated in neurons and other cell types.

Since PKC has been shown to promote the translocation of APP to the cell surface (30), we tested whether the cell surface expression of BACE1 protein was changed in PMA-treated neurons. We found that the levels of plasma membrane-bound BACE1 as well as APP was significantly increased in PMA-treated neurons compared to vehicle-treated controls. It has been documented that the BACE1-mediated cleavage of APP at the β 1 site primarily occurs in the lumen of Golgi, secretory vesicles, and endosomes, which provide the acidic environment required for the optimal proteolytic activity of BACE1 (28). To examine whether the translocation of BACE1 protein from the intracellular organelles to the plasma membrane affects its cleavage of APP, we quantified the levels of APP β and α -CTFs in both whole cell lysates and plasma membrane fraction of PMA-treated neurons. We found that the level of APP β 1-p CTF was significantly decreased in neurons following PMA treatment. Surprisingly, no APP β 1-p CTF was detected at the plasma membrane of neurons, suggesting that the cleavage of APP by BACE1 at the β 1 site occurs primarily intracellularly and the translocation of both APP and BACE1 to the cell surface is responsible for the reduction of APP β 1-p CTF and A β 1–40/42 (24) in PMA-treated neurons. Since APP β 1 CTFs are primarily generated and accumulated intracellularly, a simple increase of cell surface expression of BACE1 do not affect the production of APP β 1 CTF (7;21). Instead, the amount of BACE1 and APP remained inside cells is critical for the generation of APP β 1 CTF.

The overall levels of APP α CTF did not change in PMA-treated neurons, which is consistent with a previous *in vivo* study that the release of APP α soluble fragments was not altered in mouse brains by PMA treatment (24). Interestingly, we found that the accumulation of APP β 11 CTF in the plasma membrane fraction was significantly up regulated following PMA

treatment, which correlates with the increased presentation of APP and BACE1 at the cell surface of PMA-treated neurons, suggesting that the cleavage of APP by BACE1 at the β 11 site of A β occurs at the cell surface.

In summary, our findings provide novel mechanisms for PKC-mediated regulation of β -secretase activities in fibroblasts and neurons. It is particularly interesting that BACE1-mediated cleavage of APP at the β 1 site is restricted to intracellular compartments. Thus, the translocation of BACE1 to the cell surface is responsible for the reduced production of APP β 1 CTF and A β 1–40/42 in PMA-treated neurons(24).

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Figure 1. PMA suppressed the accumulation of BACE1 protein and the β-secretase activity in mouse fibroblasts

(A) Equal amount protein from wild type (WT) and BACE1^{-/-} (KO) fibroblast cells were immunoblotted with antibodies against BACE1 and β-actin.

(B) Treatment with PMA (10 μM) for 6 h induced a decline of BACE1-mediated β-secretase activity in fibroblasts as compared to treatment with DMSO (Con). N = 4, **p* < 0.0001 vs. vehicle-treated cells. Error bars represent SEM.

(C) Fibroblast cells were treated with DMSO (Con), PDD (an inactive analog of PMA), or different concentration of PMA.

(D) PMA suppressed the accumulation of BACE1 in a dose-dependent manner, whereas PDD or DMSO (Con) did not affect the accumulation of BACE1. $N = 5$ $*p < 0.005$ vs. vehicle-treated cells. AU stands for arbitrary unit.

(E) Fibroblast cells were treated with DMSO (Con) or 10 μ M PMA for different duration.

(F) PMA suppressed the accumulation of BACE1 in a time-dependent manner. $N = 4$, $*p < 0.005$ vs. vehicle-treated cells.

(G) Fibroblast cells were pretreated with PKC inhibitor Ro31-8220 (10 μ M) (Ro) for 30 min and then treated for additional 6 h with DMSO (Con) or 10 μ M PMA (P).

(H) Ro31-8220 (Ro) blocked the PMA-induced suppression of BACE1 in fibroblasts. $N = 4$, $*p < 0.0005$ vs. PMA treated cells.

Figure 2. ERKs inhibitor did not block the reduction of BACE1 protein induced by PMA in fibroblast cells

(A) Fibroblast cells were pretreated with ERKs inhibitor PD 98095 (20 μ M) (PD) for 30 min and then treated for additional 6 h with DMSO (Con) or 10 μ M PMA (P). β -actin was used as loading control.

(B) PD did not rescue the reduction of BACE1 protein induced by PMA in fibroblasts. $N = 3$, $*p < 0.0005$ vs. vehicle-treated cells. Error bars represent SEM.

(C) Fibroblast cells transfected with empty vector (Em), wild type BACE1 (WT) and BACE1-S498A (Mu) were treated with (+) or without (-) PMA (10 μ M) for 6 h. Cell lysates were also treated with (+) or without (-) *N*-glycosidase F prior to immunoblot. Noticeably, treatment with *N*-glycosidase F completely removed the sugar chains from immature and mature BACE1 protein, resulting in a predominant 50 kDa polypeptide.

Figure 3. Intracellular protease inhibitors restored the PMA-induced degradation of BACE1 protein in fibroblast cells

(A) Fibroblast cells were treated with lactacystin (L) or chloroquine (Ch) alone for 24h prior to immunoblot with BACE1 antibody. β-actin was used as loading control.

(B) Treatment with chloroquine (Ch) for 24 h blocked the degradation of BACE1 in fibroblasts. In contrast, treatment of lactacystin (L) for 24 h did not affect the accumulation of BACE1 protein in fibroblasts. $N = 3$, $*p < 0.00001$ vs. vehicle-treated cells. Error bars represent SEM.

(C) Fibroblast cells were pretreated with various protease inhibitors for 30 min and then co-incubated with DMSO (Con) or PMA (10 μM) (P) for 6 h. MG312 (M, 10 μM), lactacystin (L, 10 μM), and chloroquine (Ch, 50 μM) were used in this study.

(D) Pretreatment with MG312 (M) or chloroquine (Ch) blocked PMA-induced suppression of BACE1 accumulation. Meanwhile, treatment with MG312 (M), chloroquine (Ch) or lactacystin (L) alone for 6 h did not affect the accumulation of BACE1 protein in fibroblast cells. $N = 3$, $*p < 0.0005$ vs. PMA-treated cells.

(E) Fibroblast cells were pretreated with calpain inhibitor, calpeptin (Ca, 25 μ M) or cathepsin inhibitor, cathepsin inhibitor 1 (Ct, 25 μ M) for 30 min and then treated for additional 6 h with 10 μ M PMA.

(F) Pretreatment with calpeptin (Ca) or cathepsin inhibitor 1 (Ct) blocked PMA-induced suppression of BACE1. Meanwhile, treatment with calpeptin (Ca) or cathepsin inhibitor 1 (Ct) alone did not affect the accumulation of BACE1 protein in fibroblast cells. $N = 3$, $*p < 0.0005$ vs. PMA-treated cells.

Figure 4. Treatment of PMA did not affect the accumulation of BACE1 in primary cultured neurons

(A) Treatment of neurons with vehicle (Con) or PMA (10 μ M) for 3 or 6 h. Equal amount protein extracted from neurons was immunoblotted with antibodies against BACE1 and β III-tubulin.

(B) Treatment with PMA (10 μ M) for 3 or 6 h did not change the accumulation of BACE1 protein in neurons as compared with the vehicle-treated control neurons (Con). N = 5. Error bars represent SEM.

(C) Primary culture neurons were treated with MG-132 (M) or chloroquine (Ch) alone for 24h prior to immunoblot with a BACE1 antibody.

(D) Treatment with MG-132 (M) or chloroquine (Ch) did not affect the accumulation of BACE1 in neurons. N = 3.

Figure 5. PMA induced translocation of BACE1 to the cell surface in primary cultured neurons
 (A) Neurons were treated with DMSO (Con) or 10 μ M PMA for 3 or 6 h and biotinylated. The biotinylated plasma membrane-bound proteins were purified and subjected to immunoblot with BACE1 antibody. β -tubulin was used as loading control.
 (B) Plasma membrane-bound BACE1 protein was significantly increased in neurons treated with PMA as compared with vehicle-treated neurons. $N = 5$, $*p < 0.005$ vs. vehicle-treated neurons. Error bars represent SEM.
 (C) Fibroblasts were treated with DMSO (Con) or 10 μ M PMA for different duration and biotinylated. The biotinylated plasma membrane-bound proteins were purified and subjected to immunoblot with BACE1 antibody.

(D) Both cell surface-bound and total BACE1 proteins were decreased at a similar rate in PMA-treated fibroblasts as compared with vehicle-treated cells. N = 4, * $p < 0.001$ vs. vehicle-treated cells.

Figure 6. Treatment of PMA reduced the accumulation of APP $\beta 1$ CTF in cultured neurons
 (A) Neurons were treated with DMSO (–) or 10 μ M PMA for 6 h and biotinylated. The accumulation of APP full-length (APP-FL) and APP CTFs were revealed by immunoblot with an APP C-terminal antibody. β -tubulin was used as loading control.
 (B) The biotinylated plasma membrane-bound APP-FL and CTFs were purified and subjected to immunoblot with an APP C-terminal antibody.
 (C) Total APP-FL and APP CTFs were quantified and normalized by β -tubulin or APP-FL, respectively. $N = 4$, $*p < 0.02$. Error bars represent SEM.
 (D) Cell surface-bound APP-FL and APP CTFs were quantified and normalized against total APP-FL and APP CTFs, respectively. $N = 4$, $*p < 0.0001$.