Regulation of Amyloid Precursor Protein (APP) Secretion by Protein Kinase Cα in Human Ntera 2 Neurons (NT2N)†

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ABSTRACT: Cleavage of amyloid precursor protein (APP) by β-secretase generates β-amyloid (Aβ), the major component of senile plaques in Alzheimer's disease. Cleavage of APP by α-secretase prevents Aβ formation, producing nonamyloidogenic APP products. Protein kinase C (PKC) has been shown to regulate APPs secretion, and PKCα and PKCε have been implicated in APPs secretion in fibroblasts. This study examined the PKC isoform involved in regulated APPs secretion in human NT2N neurons and in CHO cells stably expressing APP_{695}. Inhibition of PMA-induced APPs secretion with the PKC inhibitors Calphostin C and GF109203X demonstrated that PKC is involved in PMA-regulated APPs secretion in NT2N cells. The specific PKC isoforms present in NT2N and CHO695 cells were identified, and PKCα and PKCε were found to translocate from cytosol to membranes in NT2N and CHO695 cells. Translocation of PKC to the membrane allows for activation of the enzyme, as well as for positioning of the enzyme close to its substrate. Long-term PMA treatment led to complete downregulation of PKCα in NT2N cells and to downregulation of PKCα and PKCε in CHO695 cells. PKCα downregulation in the NT2N cells resulted in loss of PMA-regulated APPs secretion and a substantial reduction in constitutive APPs secretion. Downregulation of PKCα and PKCε in CHO695 cells resulted in loss of PMA-regulated APPs secretion; however, constitutive APPs secretion was unaffected. These findings suggest that PKCα is involved in PMA-regulated APPs secretion in NT2N cells and PKCα and/or PKCε is involved in PMA-regulated APPs secretion in CHO695 cells.

The amyloid precursor protein (APP)[1] is a transmembrane glycoprotein that can be cleaved to generate β-amyloid (Aβ) peptide, implicated in the pathogenesis of Alzheimer's disease (AD). Aβ is found within the characteristic senile plaques of Alzheimer's disease, along with neurofibrillary tangles and dystrophic neuritic processes. APP is a family of transmembrane glycoproteins with a large extracytoplasmic domain, a membrane-spanning domain containing the Aβ peptide, and a short intracytoplasmic domain (*1*). APP is transcribed as three alternatively spliced isoforms, ranging from 695 to 770 amino acids in length, and is expressed in mammalian neuronal and nonneuronal cells and tissues (*2*). Mutations in the APP gene result in the aberrant processing of APP, which may lead to Aβ deposition and accumulation (*3*, *4*). Human familial Alzheimer's disease mutant APP transgenic mice express high levels of APP and demonstrate an age-dependent increase in Aβ (*5*). This supports the hypothesis of a primary role for APP and Aβ in the pathogenesis of Alzheimer's disease.

APP can be processed through various pathways to generate both amyloidogenic and nonamyloidogenic peptides. APP processed by a constitutive secretory pathway (or α-secretase) produces a large secreted form ($APPs_\alpha$) excluding Aβ formation (*6*, *7*). The subsequent cleavage of the membrane-bound carboxy-terminal segment by a γ-secretase produces a 3 kDa nonamyloidogenic fragment. APP processed by β- and γ-secretases or through the endosomal or lysosomal pathway results in the production of Aβ (*8*, *9*). Most recently, an aspartic protease, BACE (*b*eta-site *A*PP-*c*leaving *e*nzyme), was cloned and characterized as exhibiting β-secretase activity (*10*−*13*).

Receptor-mediated regulation of $APPs_\alpha$, such as muscarinic and metabotropic glutamate receptors, has been shown to involve the downstream participation of protein kinase C (PKC) in the secretion of $APPs_\alpha$ (*14*−*18*). Protein kinase C is a family of serine/threonine kinases involved in various functions in many cell types. The conventional PKC isoforms, α, βI, βII, and γ, are calcium-dependent and activated by diacylglycerol (DAG), phosphatidylserine (PS), and phorbol esters. The novel PKC isoforms, ε, δ, η, and θ, are calcium-independent and activated by DAG, PS, and phorbol esters. The atypical PKC isoforms, λ and ζ, are activated by PS, but do not have a calcium or DAG requirement. Certain

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[1] Abbreviations: APP, amyloid precursor protein; Aβ, β-amyloid; ADAM, a disintegrin and metalloprotease; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; NT2, Ntera 2/cl.D1; NT2N, Ntera 2/cl.D1 differentiated neurons; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; OAG, 1-oloeyl-2-acetyl-*sn*-glycerol; PAGE, polyacrylamide gel electrophoresis; TACE, tumor necrosis factor α-converting enzyme; TPA, 12-tetradecanoyl-13-acetyl-β-phorbol.

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PKC isoforms can be activated by phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), which results in the secretion of $APPs_\alpha$ (*6*, *7*, *15*, *19*). PKC activates by phosphorylation of various proteins in vivo; however, it has been demonstrated that PKC does not directly phosphorylate APP (*20*, *21*). Therefore, PKC is involved in the regulation of APP through another action. One possibility is that PKC activates one of the secretases that act on APP to generate $APPs_\alpha$. Another possibility is that PKC phosphorylates and activates proteins upstream of the secretases and APP. Phorbol ester stimulation of PKC results in the production of $APPs_\alpha$, rather than $APPs_\beta$, and a decrease in the production of Aβ (*22*−*25*). PKCα and -ϵ have been implicated in the regulation of APPs secretion from fibroblasts (*26*−*28*). However, it has not been demonstrated which PKC isoform is involved in regulated APPs secretion in neurons. Therefore, the goal of this study was to examine the role of specific PKC isoforms in APPs secretion in neurons. This study was conducted on NT2N neurons, as well as CHO cells stably expressing the neuronal form of APP, APP_{695}. NT2N neurons are post-mitotic, terminally differentiated human neurons that possess cell-surface markers consistent with neurons of the central nervous system. Pure cultures of neurons are obtained through differentiation of the stem cell population with retinoic acid, and treatment of the replated neuronal population with mitotic inhibitors to clear any contaminating nonneuronal cells. NT2N neurons provide a strong model for examining APP secretion because they endogenously express APP and secrete both APP and Aβ in response to physiological stimuli. In contrast to nonneuronal cells, NT2N neurons express APP_{695}, the major APP isoform expressed in the brain, and NT2N neurons process APP differently (*29*, *30*). In addition to the NT2N neurons, CHO cells stably transfected with APP_{695} were also examined.

EXPERIMENTAL PROCEDURES

Materials. The media and serum used to maintain the NT2N and CHO695 cultures were from Life Technologies (Grand Island, NY), and all of the mitotic inhibitors and antibiotics used in the cell culture were purchased from Sigma Chemical Co. (St. Louis, MO). Matrigel was from Collaborative Research (Bedford, MA). PMA4β and PMA4α were from Biomol (Plymouth Meeting, PA), and Calphostin C and GF109203X were purchased from Calbiochem (La Jolla, CA). [^{35}S]Methionine (1000−1500 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA). The anti-PKC antibodies were from Transduction Laboratories (Lexington, KY).

Cell Culture of NT2N Neurons. The human teratocarcinoma NTera2/c1.D1 (NT2) cells were maintained in Opti-MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (*31*). A total of 2.3×10^6 cells were seeded into 75 cm^2 flasks and treated with 10 μM retinoic acid (in DMEM media with 10% fetal bovine serum and 1% penicillin/streptomycin) twice a week for 5 weeks. Cells were then trypsinized and replated (Replate 1) into T225 flasks for 10 days in DMEM media supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and mitotic inhibitors (1 mM cytosine arabinoside, 10 mM fluorodeoxyuridine, 10 mM uridine). The neurons were then manually dislodged and plated onto poly-lysine/matrigel (Collaborative Research) coated 10 cm dishes or 6-well plates (Replate 3). Cells were fed once a week with DMEM media supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and mitotic inhibitors (1 mM cytosine arabinoside, 10 mM fluorodeoxyuridine, 10 mM uridine) for 4 weeks. This yielded a >95% pure culture of differentiated human neurons (NT2N) that could be maintained in culture for 7−8 weeks.

Cell Culture of CHO695 Cells. CHO cells stably expressing APP_{695} were provided by Dr. Virginia Lee (University of Pennsylvania). CHO695 cells were maintained in αMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine.

Identification of PKC Isoforms by Western Blotting. NT2, NT2N, and CHO695 cells were washed twice with phosphate-buffered saline, pH 7.40, and then lysed in a buffer of 10 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP40. A bicinchoninic acid (BCA) protein assay (Sigma) was used to determine the protein levels. Protein levels were adjusted, and the samples were placed in 2× sample buffer (100 mM Tris, pH 6.80, 4% SDS, 20% glycerol, and 10 mg/L bromophenol blue) supplemented with dithiothreitol. Proteins (20−40 μg/lane) were separated on 7.5% SDS−PAGE mini-gels (Biorad) at 175 V for 1 h. Proteins were then transferred to nitrocellulose paper (Hybond C; Amersham) at 100 V for 2 h. The nitrocellulose paper was blocked overnight (1% bovine serum albumin, 10 mM Tris, pH 7.40, 150 mM NaCl, and 0.1% sodium azide), and the next day, the blots were probed with specific mouse monoclonal anti-PKC antibodies (Transduction Laboratories). The blots were washed twice with 10 mM Tris, pH 7.40, 150 mM NaCl, and 0.1% sodium azide (TNA) for 10 min, once with TNA supplemented with 0.05% Nonidet P-40 for 5 min, and twice more with TNA for 10 min. The blots were then incubated for 1 h at room temperature with a rabbit anti-mouse antibody, and then washed with TNA as before. The proteins were detected with [^{125}I]Protein A, the blots were washed, and the proteins were visualized using Image Quant software on a Molecular Dynamics PhosphorImager.

Membrane and Cytosol Preparations. NT2N and CHO695 cells in 10 cm dishes were washed twice with ice-cold phosphate-buffered saline, pH 7.40, hypotonic Hepes was added, and the cells were scraped and transferred into homogenizer tubes. The cells were homogenized and placed on ice for 10 min. The homogenates were centrifuged in an airfuge at 150000*g* (30 psi) for 5 min in order to separate membranes from cytosol. The supernatants (cytosol) were collected, and the pellets, the membrane fraction, were resuspended in hypotonic Hepes. Protein levels were determined and adjusted for equalization. Samples were analyzed on 7.5% SDS−PAGE mini-gels, transferred to nitrocellulose, and probed as described before with specific anti-PKC antibodies. The proteins were detected by [^{125}I]Protein A, and visualized using Image Quant software on a Molecular Dynamics PhosphorImager.

Pharmacological Treatment of NT2N and CHO695 Cells. NT2N or CHO695 6-well plates were washed 3 times in Krebs−Hepes buffer (25 mM Hepes, pH 7.4, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 3 mM D-glucose) and then incubated under an atmosphere of 95% O_2/5% CO_2 at 37 °C for the appropriate time with 10 μM PMA. To examine

the effect of the protein kinase C inhibitors (Calphostin C and GF109203X), the cells were pretreated with the agents for 30 min at 37 °C and then incubated for an additional 30 min alone or in the presence of 10 μM PMA.

Measurement of APPs Secretion by Metabolic Labeling and Immunoprecipitation. To metabolically label APP, NT2N and CHO695 cells were serum-starved for 20 min in DMEM methionine-free media and then labeled with 400−500 μCi/mL [^{35}S]methionine in DMEM methionine-free media (1% penicillin/streptomycin, 5% fetal bovine serum) for 3 h. The cells were then treated as described above. After treatment, the supernatant was removed and centrifuged for 15 min at 15000*g* to remove any remaining cells. The supernatant was precleared with protein A−Sepharose beads and then immunoprecipitated overnight with 1−2 μg of an anti-APP polyclonal antibody (Karen) and protein A−Sepharose beads as previously described (*29*). Immunoprecipitates were analyzed on 7.5% SDS−PAGE mini-gels, and the radioactivity was quantitated using Image Quant software on a Molecular Dynamics PhosphorImager. For data analysis, the control was designated as 100% within each experiment due to the differences in radioactive counts between the controls in separate experiments.

Long-Term PMA Treatment. NT2N cells and CHO695 cells were treated with vehicle, 10 μM PMA4α, or 10 μM PMA4β for 24 h. The cells were washed twice with ice-cold PBS and lysed, and the lysate was centrifuged for 5 min at 15000*g*. The supernatants were collected and protein levels determined. Samples were analyzed by Western blotting with anti-PKC isoform-specific antibodies. For examining PMA-induced APPs secretion, the cells were metabolically labeled after 21 h, and then labeled for 3 h as stated previously with vehicle, 10 μM PMA4α, or 10 μM PMA4β present during the labeling. APPs was then immunoprecipitated from the media and quantitated as described above.

Statistical Analysis. Results were analyzed by two-way or one-way ANOVA, followed by the Bonferroni post-test, or one-way analysis of variance was performed. Differences were considered significant for $p < 0.05$.

RESULTS

Effect of PKC Inhibitors on PMA-Induced APPs Secretion. NT2N cells were labeled for 3 h with [^{35}S]methionine and then pretreated for 30 min with 1 μM Calphostin C or GF109203X. The cells were then treated with vehicle, 10 μM PMA alone or in the presence of 1 μM Calphostin C or GF109203X. Figure 1 demonstrates a 55% inhibition of PMA-induced APPs secretion from NT2N cells by Calphostin C and a 70% inhibition by GF109203X, suggesting that PKC is involved in the regulated secretion of APPs. To examine which PKC isoform(s) is (are) involved, we first needed to determine which isoforms were present in the cells.

Identification of PKC Isoforms in NT2, NT2N, and CHO695 Cells. Cell lysates were prepared from NT2, NT2N, and CHO695 cells. The NT2 cells are the population of cells differentiated with retinoic acid to produce the NT2N neurons. The proteins were analyzed by SDS−PAGE and immunoblotted with various anti-PKC isoform-specific antibodies. Figure 2 demonstrates that the conventional PKCα and the novel PKCϵ appear to be present in the NT2N and CHO695 cells and PKCγ only in the CHO695 cells. The atypical PKCλ and -ζ isoforms, and PKCμ are present in NT2, NT2N, and CHO695 cells. One way to determine PKC activity is to demonstrate the translocation of PKC from the cytosol to the membrane (*32*−*38*).

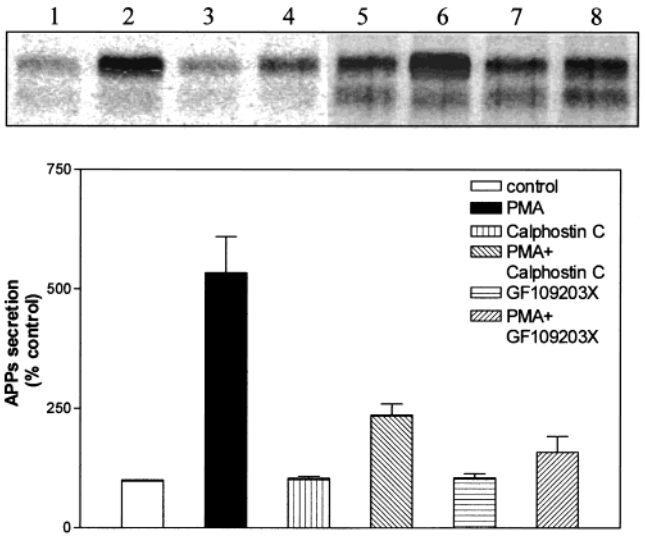

FIGURE 1: Effect of PKC inhibitors on PMA-induced APPs secretion from NT2N neurons. NT2N neurons were labeled for 3 h with [^{35}S]methionine, treated for 30 min with or without 1 μM Calphostin C or GF109203X, and then treated for 60 min with the vehicle (control) or 10 μM PMA in the presence or absence of Calphostin C or GF109203X. Top panel: Representative gel showing the APPs band in the media of NT2N neurons. Lanes 1 and 5, vehicle; lanes 2 and 6, PMA; lane 3, Calphostin C; lane 4, Calphostin C + PMA; lane 7, GF109203X; lane 8, GF109203X + PMA. Bottom panel: APPs secretion after pretreatment with Calphostin C or GF109203X and treatment with PMA ± Calphostin C or GF109203X. APPs secretion was quantitated by a PhosphorImager as the amount of radioactivity in the APPs band and is expressed as a percentage of the control within each experiment. Results are shown as the mean ± SE of APPs secretion from 2 to 4 separate observations/condition.

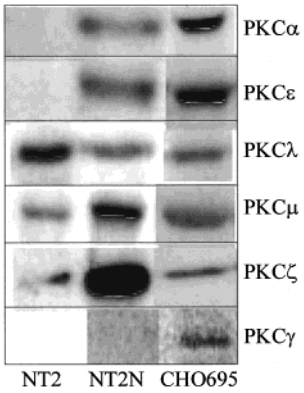

FIGURE 2: Identification of PKC isoforms in NT2, NT2N, and CHO695 cells. NT2, NT2N, and CHO695 cell lysates were analyzed by SDS−PAGE and PKC proteins detected by Western blotting with various anti-PKC isoform-specific antibodies. Representative immunoblots depicting a specific PKC band ($n = 3$−13).

PMA-Induced Translocation of PKC from the Cytosol to the Membrane. NT2N and CHO695 cells were treated with vehicle (control) or 10 μM PMA for 30 min at 37 °C. Cell homogenates were made and the cytosol/membrane fractions prepared by centrifugation of the homogenates in an airfuge at 150000*g*. Cytosol and membrane fractions were analyzed

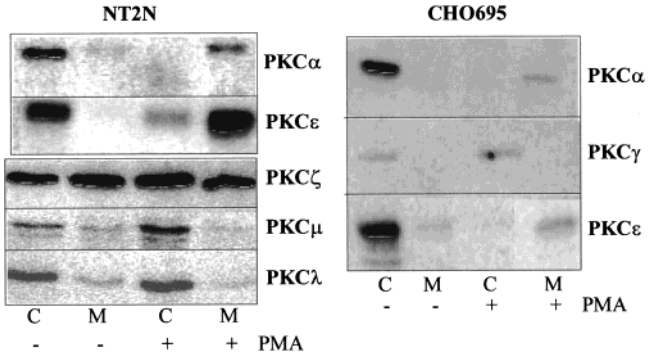

FIGURE 3: Translocation of PKCα and PKCϵ in NT2N and CHO695 cells. NT2N and CHO695 cells were treated for 30 min with or without 10 μM PMA. Cytosol (C) and membrane (M) fractions were prepared, equal amounts of the proteins were analyzed by SDS−PAGE, and PKC proteins were detected by Western blotting with various anti-PKC isoform-specific antibodies. Representative immunoblots depicting a specific PKC band within cytosol and/or membrane fractions ($n = 10$).

by SDS−PAGE and then immunoblotted with various anti-PKC isoform-specific antibodies. Figure 3 demonstrates that in both NT2N and CHO695 cells only PKCα and PKCϵ translocate from the cytosol to the membrane after PMA treatment. Both PKCα translocation and PKCϵ translocation are detectable after only 5 min of treatment with PMA, and by 30 min all of the PKCα is translocated to the membrane in both the NT2N and CHO695 cells. All of the PKCϵ is translocated to the membrane by 30 min in CHO695 cells, and the majority of PKCϵ is also at the membrane in NT2N cells. Treatment of the cells with OAG, a cell-permeable analogue of diacylglycerol, does not cause PKC to translocate from the cytosol to the membrane (data not shown). Additionally, the PKC inhibitors Calphostin C and GF109203X did not inhibit PKC translocation (data not shown). The translocation of PKC to the membrane results in its activation and positioning close to its substrate (*39*, *40*). Therefore, these results suggest that in NT2N and CHO695 cells PKCα and/or PKCϵ are involved in the PMA-regulated APPs secretion. To examine which PKC isoform(s) is (are) involved, we examined the effect of long-term PMA treatment on PKC downregulation.

Effect of Long-Term PMA Treatment on PKC in NT2N and CHO695 Cells. Studies have shown that long-term exposure of cells to phorbol esters results in the downregulation of PKC (*41*−*46*). 3T3/L1 adipocytes treated for 20 h with 16 μM tetradecanoyl-acetyl-phorbol (TPA) showed a decrease in PKCα and PKCϵ protein levels (*47*). PKCα and -ϵ downregulation has also been shown in SH-SY5Y cells after 72 h of TPA exposure (*48*). For this study, NT2N and CHO695 cells were treated with vehicle (control), 10 μM PMA4β (active), or 10 μM PMA4α (inactive analog) for 24 h at 37 °C. Cell lysates were prepared, and equal amounts of protein were analyzed by SDS−PAGE. The proteins were transferred to nitrocellulose and immunoblotted with various anti-PKC isoform-specific antibodies. Figure 4 demonstrates that long-term treatment of PMA results in the complete downregulation of PKCα in both NT2N and CHO695 cells ($p < 0.001$), and PKCϵ in CHO695 cells ($p < 0.001$), but not in NT2N neurons. PMA has only a very slight effect on PKCϵ in NT2N neurons. The long-term PMA treatment did not affect the protein levels of the other PKC isoforms including PKCζ, which was examined due to the presence of PKCζ in both membrane and cytosol fractions under basal conditions. Since long-term PMA treatment resulted in the complete reduction of only PKCα in NT2N and PKCα and -ϵ in CHO695 cells, we needed to examine regulated APPs secretion under these conditions. [Note, that constitutive APPs secretion was not changed after the 24 h of treatment (data not shown).]

Effect of PKC Downregulation on PMA-Induced APPs Secretion. NT2N and CHO695 cells were treated with vehicle (control), 10 μM PMA4β (active), or 10 μM PMA4α (inactive analogue) for 21 h at 37 °C. At that point, the cells were labeled with [^{35}S]methionine in the presence of the vehicle, PMA4β, or PMA4α for 3 h at 37 °C. The cells were then treated with the vehicle or 10 μM PMA for 30 min at 37 °C. After immunoprecipitation of the APPs from the media, the proteins were analyzed by SDS−PAGE and quantitated on a PhosphorImager. For the experiments shown, an anti-APP antibody was used that detected all species of secreted APP; however, experiments using an antibody that recognizes the APP α-secretase cleavage site demonstrated that the PMA-induced secretion is $APPs_\alpha$ (data not shown). Figure 5 demonstrates that after 24 h of treatment with the vehicle or PMA4α, the inactive analogue, PMA, is still able to induce APPs secretion in NT2N (5-fold and 4.7-fold, respectively) and CHO695 cells (5-fold and 3.4-fold, respectively). However, after 24 h of treatment with PMA, i.e., the downregulation of PKCα, PMA is not able to induce APPs secretion in NT2N neurons. Furthermore, the constitutive APPs secretion is reduced. The continued presence of PKCζ, -λ, -μ, and -ϵ after long-term PMA treatment, the absence (downregulation) of PKCα, and the inhibition of constitutive and PMA-induced APPs secretion after long-term treatment led us to conclude that PKCα was involved in the constitutive and regulated APPs secretion in NT2N neurons. In CHO695 cells, long-term PMA treatment causes the downregulation of both PKCα and PKCϵ and the loss of PMA-induced APPs secretion as shown in Figure 5. However, unlike the NT2N cells, PKC downregulation in CHO695 cells does not result in the reduction of constitutive APPs secretion. These results suggest that PKCα is responsible for the PMA-regulated APPs secretion and is involved in the constitutive APPs secretion in NT2N cells, and that PKCα and/or PKCϵ are involved in the PMA-regulated APPs secretion in CHO695 cells.

DISCUSSION

PKC is expressed in many different cell types and is involved in the regulation of many diverse cellular processes. PKC isoforms are widely distributed and are localized to different cellular compartments. PKCα, -δ, and -ζ are universally distributed, while PKCϵ and -γ are found mainly within the brain. Most of the inactive PKC isoforms are cytoplasmic; however, upon treatment with phorbol esters, PKCα, -ϵ, -γ, and -η have been shown to associate with the Golgi, cell and nuclear membranes, and/or endoplasmic reticulum (*49*, *50*). PKCα has also been localized to focal adhesions in rat fibroblasts (*51*), and along with PKCϵ, PKCα has been implicated in integrin signaling (*52*). PMA activation of PKC has been shown to stimulate the formation of transport vesicles from the trans-Golgi network and to be involved in calcium-dependent exocytosis in permeabilized cells (*53*−*56*). In addition, PKC has been shown to be

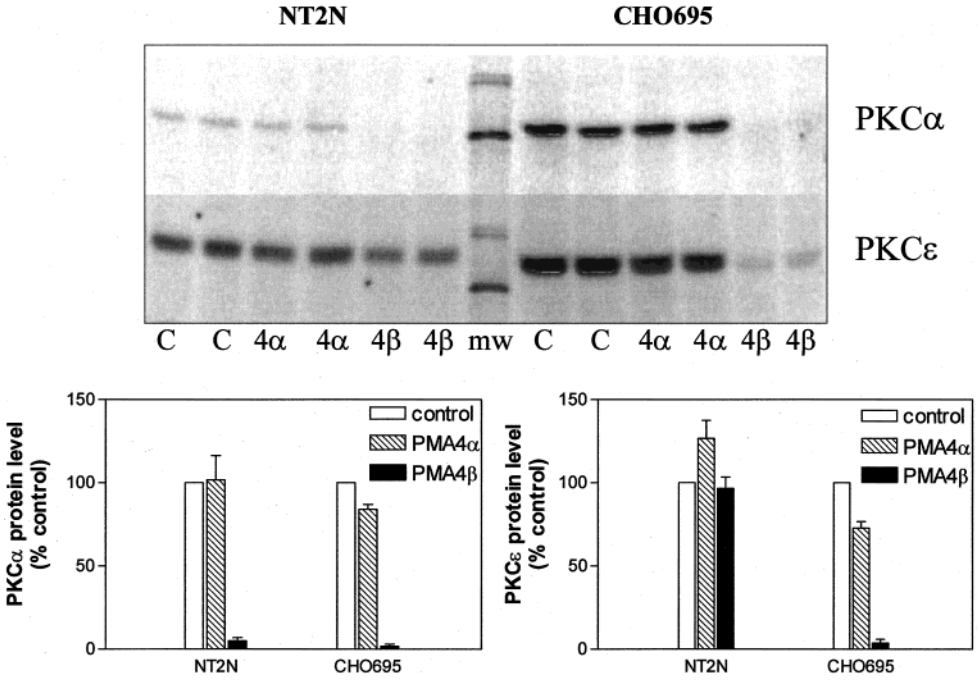

FIGURE 4: Downregulation of PKCα and PKCϵ in NT2N and CHO695 cells. NT2N and CHO695 cells were treated for 24 h with vehicle, 10 μM PMA4α (inactive), or 10 μM PMA4β. Equal amounts of proteins were analyzed by SDS−PAGE, and PKC proteins were detected by Western blotting with various anti-PKC isoform-specific antibodies. Top panel: Representative immunoblot of PKCα and PKCϵ bands. Bottom panel: PKCα and PKCϵ protein levels after 24 h of treatment with vehicle, PMA4α, or PMA4β. Results are shown as the mean ± SE of PKCα and -ϵ protein levels from 2 to 6 separate observations/condition.

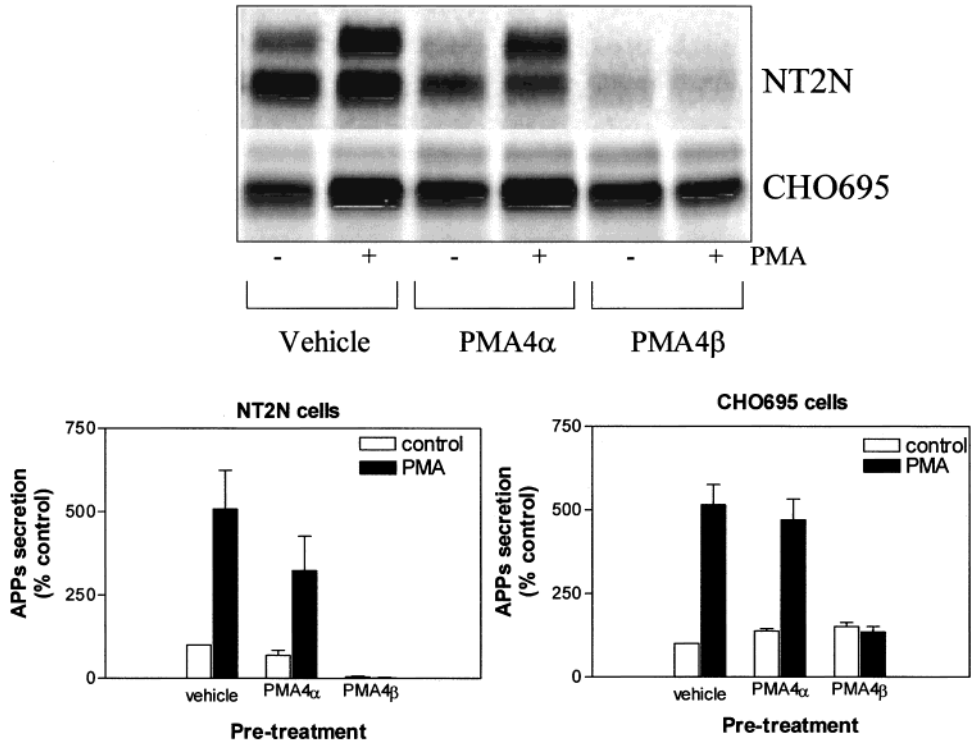

FIGURE 5: Effect of PKCα and PKCϵ downregulation on PMA-regulated APPs secretion in NT2N and CHO695 cells. NT2N and CHO695 cells were treated for 21 h with vehicle, 10 μM PMA4α (inactive), or 10 μM PMA4β. The cells were then labeled for 3 h with [^{35}S]-methionine in the presence of the vehicle, PMA4α, or PMA4β. Top panel: Representative gel of APPs secretion after treatment and immunoprecipitation with an anti-APP antibody. Bottom panel: APPs secretion after long-term treatment with vehicle, PMA4α, and PMA4β. APPs secretion was quantitated by a PhosphorImager as the amount of radioactivity in the APPs band and is expressed as a percentage of the control within each experiment. Results are shown as the mean ± SE of APPs secretion from 4 to 7 separate observations/condition.

involved in neuronal differentiation, as well as in gene expression and cell proliferation (*48*, *57*−*60*). PKC regulation of postsynaptic membrane channels and receptors has also been demonstrated (*61*, *62*). PKCα involvement in the regulation of phospholipase D and P-glycoprotein activities and expression of intracellular adhesion molecule 1 (ICAM-1) and mammary cell growth have also been demonstrated (*63*−*67*). PKCα has also been implicated in myoblast

proliferation and differentiation, and PKCα inhibition by a ribozyme led to apoptosis in glioma cells (*68*, *69*).

Regulation of APPs secretion by muscarinic and metabotropic glutamate receptors has also been shown to involve PKC through the phospholipase C-linked pathway (*14*, *15*, *17*, *18*, *70*). Activation of this pathway results in an increase in intracellular calcium, DAG, and IP_3. Calcium and DAG are then capable of activating PKC. Activation of PKC by phorbol esters results in the release of a large NH_2-terminal fragment of APP (APPs) (*6*, *7*). PKC has been shown to favor α-secretase cleavage of APP, thereby precluding the formation of Aβ, and actually decreasing Aβ generation (*24*, *25*). Recently, tumor necrosis factor α (TNF-α) converting enzyme (TACE) and ADAM-10 (*a d*isintegrin *a*nd *m*etalloprotease) were implicated in PKC-regulated α-secretase cleavage of APP (*71*, *72*). TACE cleaves the membrane-bound TNF-α precursor to release soluble TNF-α from cells. Inhibitors of TACE have been shown to inhibit basal and PMA-induced ectodomain shedding of APP, and TACE knockout mice lack the ability to induce APPs secretion with PMA (*71*, *73*). Overexpression of ADAM-10 results in an increase in PKC-stimulated APPs secretion in HEK293 cells, and expression of a dominant-negative form of ADAM-10 inhibited endogenous α-secretase activity (*72*).

Additionally, PKC results in a decrease in APP at the cell surface and therefore a decrease in the amount of internalized APP available for degradation to Aβ (*74*−*76*). Treatment of human neuroglioma cells expressing the APP Swedish mutant with phorbol dibutyrate results in the reversal of the mutant phenotype to the wild-type phenotype, thereby increasing α-secretase cleavage and decreasing β-secretase activity and Aβ production (*77*). In addition, transgenic mice also expressing the APP Swedish mutation demonstrated a decrease in Aβ production after intracortical injection of PMA (*78*). Therefore, it is important to understand the role of PKC in the regulation of APPs secretion, and for therapeutic purposes to identify the PKC isoform(s) involved.

Previous studies have implicated PKCα in regulated APPs secretion in Swiss 3T3 fibroblasts and PKCα and PKCε in 3Y1 fibroblasts (*26*−*28*). Overexpression of PKCα in Swiss 3T3 fibroblasts resulted in an increase in PMA-induced APPs secretion, and in rat 3Y1 fibroblasts, overexpression of PKCα and PKCε caused an increase in PMA-induced APPs secretion. Overexpression of PKCε also caused an increase in the basal APPs secretion. Recently, Cedazo-Minguez et al. (1999) also demonstrated the involvement of specific PKC isoforms in regulated APP processing in human neuroblastoma SH-SY5Y cells (*79*). Nicergoline, an inhibitor of acetylcholinesterase, an antagonist of α-adrenergic receptors, and an agent used in the treatment of cerebrovascular disorders and dementia, was found to stimulate APPs secretion and cause the translocation of PKCα, -γ, and -ε. Inhibitors of PKC were able to reduce the nicergoline-induced APPs secretion; however, a selective inhibitor of PKCα did not have any effect. This study implicates PKCγ and -ε in nicergoline-induced APPs secretion. These results suggest that different PKC isoforms may be involved in the constitutive versus regulated APPs secretion. A decrease in PKC activity has been detected in patients with AD, and in hippocampal tissue from AD patients, there is a decrease in the association of PKCα and PKCβ with membranes (*80*, *81*).

We have shown that inhibitors of PKC inhibit PMA-regulated APPs secretion in human NT2N neurons and have identified PKC isoforms present in NT2N and CHO695 cells. NT2N neurons provide a unique model for central nervous system neurons and endogenously express APP_{695}, the APP isoform predominantly expressed in neuronal tissue. CHO cells expressing the neuronal APP_{695} isoform were also examined. Both NT2N neurons and CHO695 cells express similar PKC isoforms, and APP processing and secretion in CHO cells have been widely studied. Additionally, cells process APP differently and demonstrate a diversity in the expression of specific proteins; therefore, it is important to examine signaling pathways in different cell types. PKCα and PKCε were shown to translocate from the cytosol to the membrane after PMA treatment in both NT2N and CHO695 cells. The downregulation of PKCα resulted in the loss of PMA-regulated APPs secretion, as well as constitutive APPs secretion in NT2N neurons. Long-term PMA treatment caused the downregulation of both PKCα and PKCε in CHO695 cells. This also resulted in the loss of PMA-regulated APPs secretion, but not in APPs constitutive secretion. These findings demonstrate an involvement of PKCα in both constitutive and regulated APPs secretion in human NT2N neurons and an involvement of PKCα and PKCε in PKC-regulated APPs secretion in CHO695 cells.

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