Protein Kinase C-dependent α-Secretase Competes with β-Secretase for Cleavage of Amyloid-β Precursor Protein in the Trans-Golgi Network*

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The release of amyloidogenic amyloid-β peptide (Aβ) from amyloid-β precursor protein (APP) requires cleavage by β- and γ-secretases. In contrast, α-secretase cleaves APP within the Aβ sequence and precludes amyloidogenesis. Regulated and unregulated α-secretase activities have been reported, and the fraction of cellular α-secretase activity regulated by protein kinase C (PKC) has been attributed to the ADAM (a disintegrin and metalloprotease) family members TACE and ADAM-10. Although unregulated α-secretase cleavage of APP has been shown to occur at the cell surface, we sought to identify the intracellular site of PKC-regulated α-secretase APP cleavage. To accomplish this, we measured levels of secreted ectodomains and C-terminal fragments of APP generated by α-secretase (sAPPα) (C83) versus β-secretase (sAPPβ) (C99) and secreted Aβ in cultured cells treated with PKC and inhibitors of TACE/ADAM-10. We found that PKC stimulation increased sAPPα but decreased sAPPβ levels by altering the competition between α- versus β-secretase for APP within the same organelle rather than by perturbing APP trafficking. Moreover, data implicating the trans-Golgi network (TGN) as a major site for β-secretase activity prompted us to hypothesize that PKC-regulated α-secretase(s) also reside in this organelle. To test this hypothesis, we performed studies demonstrating proteolytically mature TACE intracellularly, and we also showed that regulated α-secretase APP cleavage occurs in the TGN using an APP mutant construct targeted specifically to the TGN. By detecting regulated α-secretase APP cleavage in the TGN by TACE/ADAM-10, we reveal ADAM activity in a novel location. Finally, the competition between TACE/ADAM-10 and β-secretase for intracellular APP cleavage may represent a novel target for the discovery of new therapeutic agents to treat Alzheimer’s disease.

Posttranslational processing of the amyloid-β precursor pro-

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1 The abbreviations used are: APP, amyloid-β precursor protein; Aβ, amyloid-β peptide; Aβ1–40 and Aβ1–42, 40- and 42-amino acid-long forms of Aβ, respectively; AD, Alzheimer’s disease; APP751, wild-type human APP695 protein; APP695, modified to contain a dilsine ER retention motif; APPK670, APP695 containing the KM/NL familial AD Swedish mutation; ADAM, a disintegrin and metalloprotease; TACE, tumor necrosis factor-α-converting enzyme; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; αsecretase, regulated and unregulated α-secretase activities, respectively; TAPI, tumor necrosis factor-α protease inhibitor; C99, C89, and C83, the 99-, 89-, and 83-amino acid C-terminal fragments of APP, respectively; SFV, Semliki Forest virus; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TGN, trans-Golgi network; ER, endoplasmic reticulum; IC, intermediate compartment; mAb, monoclonal antibody; sAPPα, ectodomain of APP generated by α-secretase.
pressing wild-type APP. In contrast to the Swedish mutation, which increases β-secretase cleavage, activation of protein kinase C (PKC) has been shown to favor α-secretase cleavage at the expense of β-secretase cleavage. For example, treatment of cells with the PKC activator phorbol 12-myristate 13-acetate (PMA) increases secretion of sAPPα and decreases secretion of Aβ (10, 11). Transgenic mice engineered to produce high levels of human Aβ also have decreased levels of brain Aβ following PMA treatment, suggesting that stimulation of α-secretase cleavage may be a useful intervention in AD (12).

The PMA-stimulated APP cleaving activity has recently been attributed to the ADAM (a disintegrin and metalloprotease) family member tumor necrosis factor-α-converting enzyme (TACE) (13–15). TACE has been shown to cut APP at the α-secretase cleavage site in vitro, and inhibitors of TACE, such as tumor necrosis factor-α protease inhibitor (TAPI (N-(R-(2-hydroxyaminocarbonyl)-methyl)-4-methylpentanoyl-L-naphthyamine-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) proceeded for another 1.5 h. Complete growth medium was replaced and infection was allowed to occur in serum-free medium at a multiplicity of infection of 10. After 1.5 h, titered as described previously (3, 17, 18). CHO-Pro5 cells were infected with TACE knockout mice generate lower levels of sAPPα than wild-type APP. In contrast to the Swedish mutation, which increases β-secretase cleavage at the expense of β-secretase cleavage, which is regulated by PKC (19, 20). BAN-50 (a mAb specific for the first 10 amino acids of Aβ) was used as a capturing antibody, and horseradish peroxidase-conjugated BA-27 (a mAb specific for Aβ1–40) and horseradish peroxidase-conjugated BC-05 (a mAb specific for Aβ1–42) peptides (Bachem Bioscience Inc., King of Prussia, PA) were used to generate standard curves. The BAN50, BA-27, and BC-05 mAbs were prepared and characterized as described previously (20, 21).

Sandwich ELISA—Sandwich ELISA was performed as described previously using mAbs specific for different species of Aβ (19, 20). After brief sonication, medium and cell lysates were centrifuged at 40,000 rpm for 20 min at 4 °C, and both were subjected to immunoprecipitation with 6E10 (a monoclonal antibody specific for Aβ1–17), 2493 (a rabbit anti-APP antibody raised to the last 40 amino acids of APP), or Karen (a goat anti-APP extracellular domain antibody) as described previously (19).

Regulated Cleavage of APP in the TGN

Regulated Cleavage of APP in the TGN—Regulated cleavage of APP by proteinase K (PKC) has been shown to be dependent on the presence of PKC (19, 20). While reducing PMA-induced sAPPα levels to below control levels, suggests that PMA stimulates cleavage of APP by PKC at the C-terminal fragment of APP (SFV-APP TGN; a gift from A. Chyung), or an APP mutant in which a furin tail replaced the cytoplasmic domain of APP (SFV-APP ΔC; a gift from A. Chyung), or an APP mutant in which the third and fourth amino acids from the C terminus of APP have been changed to lysines (SFV-APP ΔC-KK) were prepared and titrated as described previously (17, 18, 19). CHO-Pro5 cells were infected with TACE knockout mice generate lower levels of sAPPα than wild-type APP. In contrast to the Swedish mutation, which increases β-secretase cleavage at the expense of β-secretase cleavage, which is regulated by PKC (19, 20). BAN-50 (a mAb specific for the first 10 amino acids of Aβ) was used as a capturing antibody, and horseradish peroxidase-conjugated BA-27 (a mAb specific for Aβ1–40) and horseradish peroxidase-conjugated BC-05 (a mAb specific for Aβ1–42) peptides (Bachem Bioscience Inc., King of Prussia, PA) were used to generate standard curves. The BAN50, BA-27, and BC-05 mAbs were prepared and characterized as described previously (20, 21).

Western Blot Analyses—CHO Pro 5 cells were lysed as described above. Proteins were resolved on 7.5% Tris-glycine gels and probed with a mAb raised against the catalytic domain of TACE or C-15 (goat polyclonal anti-serum raised against the C-terminal domain of TACE; Santa Cruz). Anti-mouse or anti-goat HRP-conjugated secondary antibodies were used followed by visualization with ECL.

Trypsin and Triton X-100 Treatment of Cells—Cells were treated with and without PMA for 15 min. Cells were then treated with trypsin (10 μg/ml in phosphate-buffered saline without calcium or magnesium) or with trypsin and 0.1% Triton X-100 on ice. Cells were washed in phosphate-buffered saline containing 100 μg/ml soybean trypsin inhibitor and lysed for Western blotting or immunoprecipitation as before.

RESULTS

PKC Stimulation Increases α-Secretase Cleavage of APP While Reducing β-Secretase Cleavage and Aβ Production in an ADAM-Dependent Manner—The effects of PMA and the ADAM protease inhibitor TAPI on sAPPα secretion were examined in CHO cells stably transfected with APPΔC (CHOΔC), CHOΔ95 cells express high levels of APP and constitutively secrete easily measurable amounts of sAPPα. As early as 15 min following PMA treatment, secretion of sAPPα was increased by approximately 3–5-fold (data not shown). The effects of PMA on sAPPα secretion were cumulative for approximately 1 h, but over longer time periods (3–5 h), this effect waned, and sAPPα secretion returned to baseline levels (data not shown). Therefore, we examined APP secretion during the first hour following PMA treatment by metabolically labeling CHOΔ95 cells with [35S]methionine for 1 h followed by a chase in the presence or absence of PMA (Fig. 1, A and C). sAPPα was quantitatively immunoprecipitated and visualized on Tris-glycine gels. Addition of PMA induced sAPPα secretion by approximately 3-fold. However, the protease inhibitor TAPI abolished the PMA effect and even reduced sAPPα levels to baseline levels, suggesting that TACE/ADAM-10-mediated cleavage of APP accounts not only for the PKC-regulated α-secretase activity (α(reg)) induced by PMA, but for much of the constitutive α-secretase activity as well. Because TAPI did not fully inhibit sAPPα release, another TAPI insensitive α-secretase may also cleave APP in both stimulated and unstimulated cells. To eliminate the possibility that the sAPPα recovered during the 1-h chase was produced prior to the addition of TAPI, TAPI was added as early as 1 h prior to PMA treatment. The effects of TAPI on APP secretion were maximal when TAPI was added 30 min prior to chase. No decrease in TAPI insensitive secretion was observed.
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Noted with longer pretreatments or higher concentrations of TAPI.

If β-secretase activity were limited by the availability of APP, then increased cleavage of APP by other secretases (such as α_reg) could decrease β-secretase cleavage of APP and hence Aβ production. To determine whether or not increased α_reg led to reduced β-secretase activity, we examined the effects of PMA induced ADAM activity on β-secretion of APP, using an antibody that specifically recognizes sAPPβ for immunoprecipitation (18). Fig. 1, B and D, shows that PMA reduced secretion of sAPPβ by 23%. To determine whether activation of TACE/ADAM-10 was involved in the decreased secretion of sAPPβ, we tested the effects of TAPI on sAPPβ. As shown in Fig. 1B, TAPI pretreatment restored sAPPβ secretion to baseline levels. Increased α_reg activity also reduced production of Aβ. CHO695 cells were treated with PMA and/or TAPI and levels of secreted Aβ1–40 and Aβ1–42 were quantitated by sandwich ELISA. Fig. 2 shows that PMA decreased secretion of Aβ1–40 and Aβ1–42 by 51 and 31%, respectively. In contrast, pretreatment with TAPI restored secretion of both Aβ isoforms to control levels. Taken together, these data suggest APP is a limiting substrate for which both α_reg and the β-secretase activities compete. Furthermore, because enhanced α_reg cleavage of APP reduces both β- and γ-cleavage of APP, α_reg-cleavage of APP must occur prior to or concomitant with β-secretase cleavage.

PKC-regulated α-Secretase Competes Directly with β-Secretase for Cleavage of APP—The ability of TAPI and PMA to modulate the production of sAPPα, sAPPβ, and Aβ suggested that the α_reg and β-secretases compete for limiting amounts of APP. If true, then PMA and TAPI should also affect the production of intracellular C-terminal fragments of APP produced by α- and β-secretases. CHO695 cells were labeled for 90 min followed by 45 min of chase in the presence or absence of PMA or TAPI. APP C-terminal fragments were immunoprecipitated with an antibody specific for the last 40 amino acids of APP (2493). As shown in Fig. 3, lane 1, the products of α- and β-secretase cleavage (C83 and C99, respectively) were recovered at similar levels. In addition, a band corresponding to the last 89 amino acids of APP (C89) was recovered (as determined by radiosequencing analysis), suggesting efficient cleavage of APP at residue 11 of Aβ in CHO cells. Following PMA treatment, levels of C99 and C89 decreased by 40%, whereas C83 levels increased 1.6-fold. Thus, changes in levels of intracellular C-terminal fragments of APP mirrored changes seen in sAPPα and sAPPβ levels, confirming that activation of PKC resulted in increased α-secretase cleavage of APP at the expense of β-secretase cleavage. Similar to secreted sAPPα, C83 levels were decreased by 46 ± 6% by TAPI. As before, TAPI pretreatment completely blocked the effects of PMA, decreasing C83 recovery by 53 ± 7% (Fig. 3, lane 4), eliminating the possibility that a non-metalloprotease-mediated event (such as a general effect of PMA on APP trafficking) is responsible for this effect.

To test whether the C83 generated by α_unreg and constitutively generated C83 are both substrates for γ-secretase, we treated cells with MG132, which has been shown to inhibit the intracellular γ-secretase activity that produces Aβ1–40. As expected, MG132 treatment resulted in significantly increased recovery of C99 and C83 (Fig. 3, lanes 5–8), indicating that all of these species can be turned over by a MG132-sensitive pathway, such as the Aβ1–40 producing γ-secretase. However, because MG132 is known to have other effects, such as proteasome inhibition, we cannot eliminate the possibility that these fragments are also turned over by other degradative processes. Interestingly, MG132 had similar effects on C83 levels in both unstimulated and PKC-stimulated cells, suggesting that C83 produced by α_reg and α_unreg are both turned over by a MG132-sensitive γ-secretase. Finally, TAPI pretreatment rescued C89 recovery to baseline levels in the presence of PMA, further supporting the hypothesis that α_reg competes with β-secretase for cleavage of APP.

Because α_reg-cleavage decreased β-secretase cleavage of APP, we hypothesized that α_reg and β-secretase cleavage should either occur contemporaneously or sequentially, with α_reg cleavage preceding β-secretase cleavage. Because steady-
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Fig. 3. Normal APP processing is restored by ADAM inhibition following PMA treatment. CHO cells were pulse-labeled for 90 min and chased for 45 min in the presence or absence of PMA, TAPI, and MG132. Intracellular APP C-terminal fragments were recovered from cell lysates by immunoprecipitation with 2493 and resolved on a 10%/16% discontinuous gradient Tris-Tricine gel. A representative of three experiments is shown, with bands corresponding to full-length APP, C99, C89, and C83 labeled. Contrast has been enhanced on the lower portion of the gel in order to emphasize the APP C-terminal fragments.

Fig. 4. Stimulation of PKC with PMA decreases β-secretase cleavage of APP due to competition between α-secretase and β-secretase for APP. CHO cells were pulse-labeled with and without PMA for 10 min and chased with and without PMA for the indicated times. Intracellular APP C-terminal fragments were immunoprecipitated with 2493 and resolved on discontinuous gradient Tris-Tricine gels. Representative of three experiments is shown, with bands corresponding to full-length APP, C99, C89, and C83 labeled. Contrast has been enhanced on the lower portion of the gel in order to emphasize the APP C-terminal fragments.

state labeling experiments cannot address the temporal sequence of α- and β-secretase cleavage of APP, we used a pulse-chase paradigm to test this hypothesis. To do this, CHO cells were pulse-labeled for 10 min in the presence or absence of PMA, and C-terminal APP intracellular fragments were immunoprecipitated at various time points during the chase period. Due to the low levels of C99 produced under these conditions, we used CHO cells that express the APPNL mutant, which is cleaved more efficiently than APPWT by β-secretase. As shown in Fig. 4, C99 and C83 were made contemporaneously in the absence of PMA. PMA treatment shifted APP processing away from β-secretase cleavage and toward α-secretase cleavage (as evidenced by C83 accumulation at the expense of C99), although without altering the total level of C-terminal fragment generation. This argues against a PMA induced global alteration in APP processing (such as would be expected from alterations in APP trafficking) and supports direct competition between these secretases. Importantly, even with favored β-secretase cleavage of APP in CHO cells, C99 was not produced prior to C83, arguing against the possibility that PMA stimulation results in C99 turnover to C83.

αreg Cleavage of APP Occurs in the Golgi Compartment—Because we established that αreg competes with β-secretase to cleave APP and because β-secretase activities have been detected in the ER and the TGN, we sought to identify the site of αreg cleavage. To do this, we utilized two organelle-specific APP targeting constructs: APPAKK and APPTTGN. APPAKK is an APP construct that has been modified to contain the dilyssine ER retention motif; expression of APPAKK in cells restricts APP to the ER/IC. APPTTGN is an APP/furin hybrid that contains the extracellular and transmembrane domains of APP, fused to the cytoplasmic domain of rat furin.2 The cytoplasmic domain of furin is sufficient for targeting furin to the TGN (22) and serves to target APPTTGN to the TGN as well. CHO cells infected with SFV-APPWT, SFV-APPTGN, or SFV-APPASKK were metabolically labeled with [35S]methionine for 1 h, pretreated with TAPI for 30 min, and chased with or without PMA for an additional 45 min. sAPPα was immunoprecipitated with 6E10, resolved on Tris-glycine gels, and quantitated using a PhosphorImager (Fig. 5A). In order to standardize results for varying levels of SFV infection, sAPPα levels were normalized to levels of full-length APP (Fig. 5, B and C).

As shown in Fig. 5A, infection of cells with SFV-APPASKK completely abrogated both constitutive and PMA-stimulated sAPPα secretion, suggesting a post-ER/IC locus for both α-secretases. In contrast, infection of cells with SFV-APPTGN resulted in a 49% decrease in sAPPα secretion as compared with cells infected with SFV-APPWT. This decrease could be due to increased β-secretase cleavage in the TGN because this is the major site that produced secreted Apβ. Alternatively, the decrease in sAPPα secretion could be explained if the constitutively active α-secretase were located in a post-TGN compartment. Indeed, following TAPI treatment, sAPPα secretion was reduced by 85% in APPWKTL-expressing cells, indicating that αreg accounted for the bulk of α-secretase activity in the TGN and that most of the non-TACE/ADAM-10-mediated α-secretase activity is confined to a post-TGN locus. Following PMA stimulation, sAPPα secretion was increased to similar extents in APPWKTL and APPWT-expressing cells. Because APPWKTL is predominantly retained in the TGN, this suggests that αreg cleavage of APP occurs in the TGN. In contrast, in the absence of PMA treatment, α-secretase cleavage of APP occurs both in the TGN, where TACE/ADAM-10 is required for cleavage, and post-TGN, where cleavage of APP is TACE/ADAM-10-independent.

In order to confirm that the TGN is the site in which αreg competes with β-secretase for cleavage of APP, we quantitated production of intracellular and secreted Apβ species in CHO cells expressing APPWT, APPTKNL, and APPASKK (Fig. 6). PMA attenuated Apβ1–40 secretion by a TAPI-sensitive mechanism in both APPWT- and APPTTGN-expressing cells. This further confirms that αreg and β-secretase both can cleave APP in the TGN. Interestingly, PMA treatment also decreased levels of intracellular Apβ1–40 but not intracellular Apβ1–42. This may reflect the different sites of production for intracellular Apβ1–40 and Apβ1–42 because Apβ1–40 is largely produced in the TGN, whereas intracellular Apβ1–42 is produced in the ER/IC (3, 4). Indeed production of intracellular Apβ1–42 by ER retained APPASKK was unaffected by PMA or TAPI.

Mature TACE Is Found Intracellularly—Because experi-

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1 Chyung, A. C., Lee, V. M.-Y., and Doms, R. W., manuscript in preparation.
Regulated Cleavage of APP in the TGN

In order to test whether PMA stimulation induced translocation of TACE to the cell surface, cells were pretreated with PMA for 15 min prior to trypsinization and quantitated by Western blot as before. PMA treatment did not alter the distribution or maturation of TACE (data not shown), thus indicating that even following PKC activation TACE remains intracellular.

Because TACE contains six potential N-linked glycosylation sites, it can be variably glycosylated, thus altering gel migration (14). In order to confirm the identity of the bands shown in Fig. 7, we documented TACE immunoreactive bands of similar molecular weights in THP-1 monocytes, a cell line previously shown to express abundant TACE (14). Further, antibodies raised against the catalytic domain of TACE and against the C-terminal domain of TACE both detected the ~85- and ~110-kDa bands (data not shown).

In order to test whether PMA stimulation induced translocation of TACE to the cell surface, cells were pretreated with PMA for 15 min prior to trypsinization and quantitated by Western blot as before. PMA treatment did not alter the distribution or maturation of TACE (data not shown), thus indicating that even following PKC activation TACE remains intracellular.

DISCUSSION

In a subset of familial AD cases, increased \( \beta \) production induced by mutations in the APP gene appears to be sufficient to cause AD. Thus, insights into the regulation of APP cleavage are crucially important to understanding the pathogenesis of AD. The APP ectodomain is cleaved by \( \alpha \)-secretase between residues 16 and 17 of \( \beta \), thereby precluding production of amyloidogenic \( \beta \). Whereas cells constitutively cleave APP at this site, \( \beta \)-secretase cleavage is dramatically up-regulated following treatment of cells with PKC activators such as PMA. The data presented here as well as in two other recent reports (13, 16) have implicated TACE and ADAM-10 as the \( \alpha \) enzymes because the activity of both of these enzymes is increased following PKC activation.

Currently, it is unclear whether TACE or ADAM-10 is the major \( \alpha \) enzyme, because both TACE and ADAM-10 have been demonstrated as the \( \alpha \) enzymes that cleaves APP. For example, although experiments using cells generated from knockout animals showed that TACE could account for all of the regulated secretion of APP, overexpression of ADAM-10 is sufficient to increase regulated cleavage of APP under some circumstances (13, 16). Thus, TACE may be the physiological catalyst for APP\'s secretion, but when ADAM-10 is abundant, it can also cleave APP. However, the high degree of homology between these two enzymes makes it difficult to distinguish their activities, especially because they are inhibited by similar compounds (16, 24), and mutant forms of either one may have a dominant negative effect on the other wild-type enzyme (14).

In addition to increasing \( \alpha \)-secretase cleavage, PKC activation also leads to decreased \( \beta \)-secretase cleavage of APP. How might PKC activation increase \( \alpha \)-secretase cleavage of APP and decrease \( \beta \)-secretase cleavage of APP? Previous studies have suggested that PKC may alter APP trafficking by increasing movement of APP out of the TGN and to the cell surface via secretory vesicles (23). However, our data support a role of PKC in the activation of \( \alpha \)-secretase, and the evidence is as follows. First, if \( \beta \)-secretase activity were present in the TGN, and \( \alpha \)-secretase were present at the cell surface, redistribution of APP from the TGN to the plasma membrane would result in increased \( \alpha \)-secretase cleavage and decreased \( \beta \)-secretase cleavage. However, we did not observe any redistribution of APP to the cell surface following PMA treatment. Second, we did not detect any changes in APP trafficking by pulse-chase analysis upon PKC activation (Fig. 4). Instead, we found that PKC activation led to increased enzymatic activity of \( \alpha \)-secretase and that this activity competes with \( \beta \)-secretase (which has previously been localized to the TGN) for cleavage of APP. Finally, our observation that the PMA-induced reduction in secretion of sAPP\( \beta \) and \( \beta \) was completely blocked by TAPI indicates that PMA affects APP processing by activating TAPI-sensitive metalloprotease(s) rather than by altering APP trafficking.

However, we observed incomplete inhibition of \( \alpha \)-secretase by TAPI, which could arise from the contribution of TAPI-insensitive metalloproteases to \( \alpha \)-secretase or from incomplete inhibition of TACE/ADAM-10 by TAPI. Because fibroblasts derived from TACE knockout mice do not show any TAPI-insensitive \( \alpha \)-secretase (13), it is likely that the small amount of residual \( \alpha \)-secretase activity in the presence of TAPI reflects incomplete inhibition of TACE. Thus, these data further support that TACE is the enzyme that is responsible for the majority of \( \alpha \)-secretase activity in our system.

The implication of ADAM family members in the \( \alpha \)-secretase cleavage of APP allowed us to examine the dynamic relationship between \( \alpha \)-secretase cleavage of APP to generate sAPP\( \alpha \) and \( \beta \) and \( \beta \)-secretase cleavage to generate sAPP\( \beta \) and \( \beta \). We observed an inverse relationship not only between sAPP\( \beta \) and sAPP\( \alpha \) secretion, but also between recovery of C83 and C99, the products of \( \alpha \)- and \( \beta \)-secretase activities, respectively. This inverse relationship of \( \alpha \)-secretase and \( \beta \)-secretase activities suggested that TACE/ADAM-10 and \( \beta \)-secretase may compete to cleave limiting amounts of APP (as illustrated in Fig. 8). Significantly, in the absence of PMA, CHO\( \alpha \)-secretase cells produce similar amounts of C83 and C99, but PMA increased C83 recovery while decreas-

![Diagram](image.png)

**Fig. 5.** PMA-stimulated, ADAM-dependent \( \alpha \)-secretase cleavage of APP occurs in the TGN. SFV-infected CHO cells engineered to express APP\( \text{WT} \), APP\( \text{TGN} \), or APP\( \text{ADAM} \) were pulse-labeled for 60 min and chased for 45 min in the presence or absence of PMA and TAPI. A, sAPP\( \alpha \) was recovered from the medium by immunoprecipitation with 6E10 and resolved on 7.5% Tris-glycine gels. B, intracellular APP was recovered from cell lysates by immunoprecipitation with Karen antibodies raised against the catalytic domain of TACE and against the N-terminus of APP. C, sAPP\( \alpha \) levels were quantitated and normalized for intracellular APP levels. Representative gels, means, and S.E. of three separate experiments are shown. Single factor analysis of variance revealed \( p < 0.05 \) (*) and \( p < 0.01 \) (**).
ing C99 recovery, and this effect was inhibited by TAPI, further confirming that the competition occurred via a TACE/ADAM-10-dependent mechanism. Alternatively, these data could be explained by sequential cleavage of APP by \( \beta \)-secretase (generating C99) and then by \( \alpha \)-reg (generating C83). If so, then PMA stimulation would result in increased C83 production coupled with increased C99 turnover. Two lines of evidence suggest that this is not the case. First, although \( \alpha \)-reg cleavage of C99 could explain both decreased recovery of C99 and decreased production of A\( \beta \), it could not explain the parallel decreases in sAPP\( \beta \) observed with PMA treatment. Second, \( \alpha \)-reg turnover of C99 is unlikely because pulse-chase analysis of APP C-terminal fragments showed the contemporaneous production of C99 and C83 in CHO cells. PMA treatment did, however, result in decreased levels of total cellular C99 (Fig. 3). Because PMA treatment decreases C99 levels even in the presence of MG132, which has been shown to inhibit \( \gamma \)-secretase cleavage of this species (25–27), this suggests that activated \( \alpha \)-reg competes successfully with \( \beta \)-secretase for cleavage of APP before \( \gamma \)-secre-
tase cleavage.

The results of our experiments also provide insights on aspects of β-secretase(s) activities. For example, recent reports suggest that APP<sub>ΔNL</sub> and APP<sub>WT</sub> may be cleaved by distinct β-secretases (17, 27, 28). Because the β-secretase that cleaves APP<sub>ΔNL</sub> may be localized in different organelles we asked whether α<sub>reg</sub> similarly competes with this secretase. Pulse-chase analysis showing contemporaneous production of C99 and C83 from APP<sub>ΔNL</sub> and the successful competition away from β-secretase cleavage with stimulation of α<sub>reg</sub> support the idea that the APP<sub>ΔNL</sub> cleaving β-secretase most likely also reside in the same organelle as α<sub>reg</sub>. Another characteristic of β-secretase revealed by our experiments is that although increasing α<sub>reg</sub>-cleavage caused decreased β-secretase cleavage of both APP<sub>WT</sub> and APP<sub>ΔNL</sub> decreasing α<sub>reg</sub>-cleavage did not increase β-secretase cleavage beyond baseline levels. This suggests that β-secretase cleavage is not limited by substrate availability under steady-state conditions and that only after PKC stimulation is β-secretase substrate limited.

Previous studies have shown that the TGN is a major locus for β-secretase cleavage, and our data are consistent with α<sub>reg</sub> competing with β-secretase for cleavage of APP in this organelle (Fig. 8). Indeed, our observation that APP targeted to the TGN was not efficiently cleaved by the non-ADAM-dependent β-secretase (α<sub>reg</sub>-secretase activities). For example, glutamate and the muscarinic agonist carbachol have both been shown to increase α<sub>reg</sub>-cleavage of APP (31–33). Although these agents may work through the common pathway of PKC activation, it is unclear how PKC in turn activates TACE. One possibility is that PKC directly phosphorylates TACE. Indeed, the ADAM family member MDC9 is phosphorylated following PMA stimulation (24). However, the role of PKC in ADAM phosphorylation and the consequences of such phosphorylation on protease activity are still unknown. Interestingly, the mitogen-activated protein kinase kinase inhibitor PD98059 and an inactive extracellular signal-regulated protein kinase mutant have been shown to partially antagonize regulated α-cleavage of APP (34). Whether extracellular signal-regulated protein kinase is involved in regulating ADAM activity or whether it plays a role in trafficking APP or ADAM to the TGN remains to be determined. Future studies will define further how PKC increases the activity of TACE/ADAM-10, and such information may be useful in the discovery of new therapeutic strategies for the treatment of AD.

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