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Enhanced generation of Alzheimer's amyloid-β following chronic exposure to phorbol ester correlates with differential effects on alpha and epsilon isozymes of protein kinase C

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Abstract

Alzheimer's amyloid precursor protein (APP) sorting and processing are modulated through signal transduction mechanisms regulated by protein phosphorylation. Notably, protein kinase C (PKC) appears to be an important component in signaling pathways that control APP metabolism. PKCs exist in at least 11 conventional and unconventional isoforms, and PKCα and PKCε isoforms have been specifically implicated in controlling the generation of soluble APP and amyloid-β (Aβ) fragments of APP, although identification of the PKC substrate phospho-state-sensitive effector proteins remains challenging. In the current study, we present evidence that chronic application of phorbol esters to cultured cells in serum-free medium is associated with several phenomena, namely: (i) PKCα down-regulation; (ii) PKCε up-regulation; (iii) accumulation of APP and/or APP carboxyl-terminal fragments in the *trans* Golgi network; (iv) disappearance of fluorescence from cytoplasmic vesicles bearing a green fluorescent protein tagged form of APP; (v) insensitivity of soluble APP release following acute additional phorbol application; and (vi) elevated cellular APP mRNA levels and holoprotein, and secreted Aβ. These data indicate that, unlike acute phorbol ester application, which is accompanied by *lowered* Aβ generation, chronic phorbol ester treatment causes differential regulation of PKC isozymes and *increased* Aβ generation. These data have implications for the design of amyloid-lowering strategies based on modulating PKC activity.

Keywords

amyloid β peptide; green fluorescent protein; amyloid precursor protein; phorbol-12,-14-di-butyrate; protein phosphorylation; *trans* Golgi network; vesicle incorporation

Alzheimer's amyloid precursor protein (APP) processing is complex, involving several phosphorylation-regulated pathways (Caporaso *et al.* 1992; Gandy and Greengard 1994; da

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Cruz e Silva and da Cruz e Silva 2003; Lee *et al.* 2003; Lanni *et al.* 2004; Vingtdeux *et al.* 2005). APP molecules traversing the non-amyloidogenic constitutive secretory pathway are cleaved within the amyloid-β (Aβ) domain by α-secretase (Weidemann *et al.* 1989; Esch *et al.* 1990) and the large APP ectodomain (designated soluble APPα; sAPPα) is released into the medium. Alpha-secretase cleavage of APP molecules within the Aβ sequence prevents amyloidogenesis. Alternatively, in the amyloidogenic pathway, APP is cleaved N terminally to Aβ by β-secretase (Seubert *et al.* 1993; Vassar *et al.* 1999), and the resulting amyloidogenic carboxyl terminal fragment (Gandy *et al.* 1992; Golde *et al.* 1992; Knops *et al.* 1992; Tamaoka *et al.* 1992) can subsequently undergo further proteolysis to yield Aβ (Haass *et al.* 1992; Shoji *et al.* 1992; Seubert *et al.* 1993). Stimulation of protein kinase C (PKC) by phorbol esters is one of the most robustly reproducible methods for activating sAPPα production (Buxbaum *et al.* 1992; Caporaso *et al.* 1992) and concomitantly inhibiting generation of the amyloidogenic Aβ fragment (Buxbaum *et al.* 1993; Gabuzda *et al.* 1993; Hung *et al.* 1993). Mechanistically, PKC has been shown to phosphorylate APP both *in vitro* (Gandy *et al.* 1988; Suzuki *et al.* 1992) and *in vivo* (Oishi *et al.* 1997) on Ser655 within the cytoplasmic domain (APP695 isoform numbering). However, phorbol ester stimulated release of sAPPα can occur even in the absence of the cytoplasmic tail (da Cruz e Silva *et al.* 1993), excluding an obligatory role for direct APP phosphorylation by PKC as a crucial reaction tightly linked to activation of α-secretase cleavage of APP and release of sAPPα. Alternatively, as PKC regulates formation of APP-bearing transport vesicles at the *trans* Golgi network (TGN) (Xu *et al.* 1995), one popular formulation is that PKC activates α-secretase cleavage of APP by enhancing delivery of APP out of the TGN and to the plasma membrane, where the ADAM (a disintegrin and metalloproteinase) family α-secretases ADAM-10 and ADAM-17 are concentrated. On the other hand, PKC has also been implicated in regulation of APP transcription (Trejo *et al.* 1994), an event that would likely increase generation of Aβ, thereby potentially neutralizing any therapeutic amyloid-lowering effect of chronic PKC activation. Therefore, we conducted the current study to determine how activation of α-secretase and enhancement of APP transcription might be integrated in the face of chronic exposure to a phorbol ester.

Protein kinases Cα, β, and ε have all been reported to play a role in APP processing (Benussi *et al.* 1998; Yeon *et al.* 2001; Zhu *et al.* 2001; Racchi *et al.* 2003). The specific PKC isoform(s) involved in APP metabolism vary according to the exact stage of the processing being considered, and, as noted above, PKC may regulate effects that oppose each other. For example, PKCε can stimulate basolateral endocytosis in intestinal epithelia, while PKCα can oppose this effect by stabilizing F-actin (Song *et al.* 2002). PKCα is thought to regulate APP secretion (Benussi *et al.* 1998), as cells transfected with antisense PKCα cDNA exhibited reduced sAPP production in response to phorbol esters (Racchi *et al.* 2003). Similarly, PKCε has also been implicated in APP processing (Yeon *et al.* 2001; Zhu *et al.* 2001). The use of isoform-specific PKC inhibitors indicates that PKCε is probably involved in both the activation of sAPP production and the suppression of Aβ production. In agreement with these findings, transfection with the cDNAs for either the α or the ε isoform of PKC produces corresponding increases in sAPP production, whereas the use of PKCδ fails to elicit a similar effect (Kinouchi *et al.* 1995). Also of note is the finding that down-regulation of PKCα and ε in SH-SY5Y neuroblastoma cells by the use of antisense cDNAs leads to an impairment in sAPPα-induction by phorbol esters (Yeon *et al.* 2001; Zhu *et al.* 2001). It is worth noting that the PKCα isoform has been observed to translocate from the cytosol to the plasma membrane via newly budded transport vesicles at the TGN, thus becoming ideally positioned to act upon potential PKC substrate effectors (Lanni *et al.* 2004). Interestingly, Alzheimer patients are reported to express lower levels of PKCε when compared with controls. PKCβI (Rossner *et al.* 2001) and PKCβII (Paola *et al.* 2000) may also be involved in APP processing, having been reported to modulate the intracellular levels of Aβ.

Phorbol esters exert effects not only on APP processing, but also on APP expression. Phorbol 12-myristate 13-acetate was shown to increase APP mRNA levels by inducing known components of the transcription factor activator protein-1 (AP-1) and increasing protein-DNA binding activity to AP-1 sequences within the APP promoter (Trejo *et al.* 1994). It was suggested that the distal but not the proximal AP-1 recognition site binds nuclear proteins regulated by PKC, and that the AP-1 binding activity is likely to be composed of Jun-Jun homodimers rather than Jun-Fos heterodimers (Trejo *et al.* 1994). Thus, cellular mediators that activate PKC may up-regulate expression of the APP gene and consequently affect its production and processing.

Our present findings confirm that long-term exposure to phorbol ester leads to an increase in the levels of APP, and we find that this is associated with an up-regulation of PKCε but not other isoforms. Indeed, long-term exposure of cells to phorbol-12,-14-di-butyrate (PDBu) resulted in the down-regulation of the PKCα isoform. As PKCα principally acts on APP processing and not on APP expression, we propose that this down-regulation of PKCα led to APP retention in the TGN, thereby reducing incorporation of APP into visible vesicles. Consistent with this formulation, the levels of Aβ production were significantly *increased* when PKCα was down-regulated. The long-term effects of subjecting cells to aberrant PKCα and/or PKCε environments are discussed, and the implications for targeting PKC modulation as an amyloid-lowering therapy or a prophylactic for Alzheimer disease are presented.

Materials and methods

Sample preparation and immunoblotting

Monkey kidney (COS) cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Amador *et al.* 2004). Monolayers of COS cells at approximately 80% confluency were treated with 1 μM PDBu in serum-free DMEM (sf medium). Parallel experiments were carried out in complete, serum-containing DMEM (c medium). Cells were harvested every 2 h into 1% sodium dodecyl sulfate (SDS), samples were boiled, and the protein content of the cell lysates was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA). Samples were separated by electrophoresis through 7.5% polyacrylamide gels in Tris–glycine buffer containing 0.1% SDS–polyacrylamide gel electrophoresis). Proteins resolved by SDS–polyacrylamide gel electrophoresis were electrophoretically transferred to nitrocellulose membranes. Immunoblotting of the transferred proteins was performed by first blocking possible non-specific binding sites with non-fat dry milk in 10 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl. Incubation with the primary antibody was carried out for periods from 2 h to overnight, depending upon the antibody used. Detection was achieved using the enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ, USA) or a colorimetric (Nitro-Blue Tetrazolium/5-Bromo-4-Chloro-3′-Indolyl Phosphate) system (Rebelo *et al.* 2004). For monitoring production of sAPPα, the conditioned media were collected and SDS was added to yield a final concentration of 1%. The media thus obtained are referred to as cumulative media (2, 4, 6, 8, and 10 h), and the volume loaded on the gel was normalized against the protein concentration of the corresponding cell lysate.

Immunodetection

Protein kinase C immunodetection was carried out using isozymespecific antibodies (Gibco, BRL, Invitrogen, Carlsbad, CA, USA) and the conditions specified by the manufacturer. As a control, a mouse monoclonal anti-tubulin antibody (Zymed, South San Francisco, CA, USA) was used to standardize protein loading. Other primary antibodies included the mouse

monoclonal antibody 22C11 (Roche Diagnostics GmbH, Mannheim, Germany), which binds an epitope on the extracellular domain of APP, to detect APP and both sAPPα and sAPPβ, and the mouse monoclonal antibody 6E10 (Sigma-Aldrich), which recognizes the N-terminus of Aβ either within Aβ or within sAPPα. Secondary antibodies included horse-radish peroxidase-linked anti-mouse IgG (Amersham Pharmacia) and alkaline phosphatase conjugated anti-mouse IgG (Sigma-Aldrich).

Metabolic labeling and immunoprecipitation

For metabolic labeling, cells were plated at approximately 80% confluency onto six-well culture dishes. Cells were washed twice in methionine-free DMEM, and pre-incubated for 15 min in the same solution. Cells were maintained in the presence of methionine-free DMEM for 1 h supplemented with [^{35}S]methionine (NEN-DuPont, Boston, MA, USA) during the `pulse' period. Cells were then `chased' in DMEM without serum (sf) for varying periods of time and processed for analysis. Procedures were carried out in the presence or absence of PDBu, as indicated. For studies addressing PKC-regulated APP processing, only the media corresponding to the last hour of the experimental conditions were collected. Subsequently, samples were immunoprecipitated with 6E10 (Sigma-Aldrich) after removal of cell debris by centrifugation (10 000 *g*). For experiments designed to measure secreted Aβ, media were collected after 1, 4, or 8 h and subjected to immunoprecipitation with 6E10. Immunoprecipitates were analyzed on 10–20% Tris/tricine gradient gels. Gels were treated with 2,5-diphenyloxazole/dimethyl sulfoxide and dried for fluorography. The dried gels were exposed to X-ray film, and the immunoprecipitated material was quantified as described below.

Quantification and statistical analysis

Immunoblots and autoradiograms were scanned and quantified using Quantity One densitometry software (Bio-Rad Laboratories, Hercules, CA, USA). Data are expressed as mean ± SEM of triplicate determinations, from at least three independent experiments. Statistical significance analysis was conducted by one-way ANOVA followed by the two-tail Student's *t*-test. Aβ detection from metabolically labeled immunoprecipitates was carried out using a Molecular Imager (Bio-Rad Laboratories).

Production and subcellular localization of APP-GFP fusion proteins

Fusion constructs expressing human APP695-cDNA fused to the green fluorescent protein (GFP) were prepared using standard recombinant DNA techniques. In brief, human APP695 cDNA was amplified by the PCR using specific oligonucleotide primers designed to remove the APP stop codon. The amplified fragment was then subcloned into the pEGFP-N1 mammalian expression vector (Clontech Laboratories Inc, Mountain View, CA, USA) and fully sequenced prior to use (da Cruz e Silva *et al.* 2004). Constructs bearing the Swedish mutation were also prepared. Human APP695–GFP fusion constructs were transfected into COS-7 cells previously plated onto coverslips pre-treated with 100 μg/mL poly-ornithine. The cells were treated with phorbol esters as described above and the intracellular localization of APP–GFP was determined using epifluorescence microscopy. For co-localization studies Texas Red-labeled anti-syntaxin 6 antibody (BD Biosciences, San Jose, CA, USA) was used as a TGN marker, and an anti-clathrin antibody (ICN Immunobiologicals, Aurora, OH, USA) was used as a marker of clathrin-coated vesicles.

Northern blot analysis of amyloid precursor protein total RNA

COS-7 cells were exposed for 8 h to 1 μM PDBu or vehicle in sf or c medium, as described above. Cells were lysed using TRI REAGENT (Sigma-Aldrich), and total RNA was isolated according to manufacturer's instructions. Mass-normalized aliquots (15 μg) were separated

by formaldehyde gel electrophoresis and transferred to nitrocellulose filters. The blot was hybridized with a [^{32}P]-labeled APP cDNA probe (25 ng, 1×10^6 cpm/ng), as described in the MTN Blot User Manual (BD Biosciences, Clontech). The APP probe used (756 bp) was obtained by *Age*I/*Bam*HI restriction enzyme digestion of APP751 cDNA, and labeled with [α-^{32}P]dCTP (Amersham Pharmacia, GE Healthcare, Little Chalfont, UK) using the High Prime DNA Labelling Kit (Roche Diagnostics GmbH), followed by purification through NucTrap Probe Purification Columns (Stratagene, La Jolla, CA, USA and Alfagene). Hybridizing RNAs were detected using a Molecular Imager (Bio-Rad Laboratories).

Immunofluorescence

COS-7 cells grown on coverslips and transfected with APP695–GFP, as described above, were fixed with 4% *p*-formaldehyde, permeabilized with methanol for 2 min and blocked in 3% bovine serum albumin in phosphate-buffered saline for 1 h. Co-localization studies were carried out with incubation in primary antibody to PKCα or PKCε (Gibco, BRL) and sequentially with syntaxin 6 or clathrin, respectively. Alexa 350 and Texas Red-conjugated antibodies (Molecular Probes, Eugene, OR, USA and BD Biosciences) were added for 2 h at 22°C. Between each stage of preparation, cells were carefully washed with phosphate-buffered saline. Visualization was carried out using a Zeiss LSM 510-Meta confocal microscope (Carl Zeiss Microimaging GmbH, Jena, Germany), and a 63×/1.4 oil immersion objective. Argon laser lines of 405 and 488 nm were used to excite Alexa Fluor 350 and enhanced GFP, respectively, and a 561 nm Diode Pumped Solid State laser was used to excite Texas Red. Microphotographs were acquired in a sole section in the *z*-axis (*xy*-mode) and represent a mean of 16 scans.

Results

PKC isozyme expression profiles following exposure to phorbol ester

Long-term exposure to the phorbol ester PDBu is a standard treatment used to down-regulate PKC. Thus, COS-7 cells were exposed to PDBu for increasing periods of time, and levels of PKC expression were monitored. Two distinct conditions were tested, whereby cells were either incubated in medium without serum (sf medium) or in medium with serum (c medium).

We observed that PKCα levels were down-regulated under both conditions (Fig. 1a and b). In contrast, PKCε was down-regulated only when cells were incubated in c medium (Fig. 1b), and PKCε was up-regulated when the experiment was carried out in sf medium (Fig. 1a).

Interestingly, under conditions where both PKC isozymes were down-regulated (c medium), their rates of decline were different. PKCα down-regulation was approximately linear with time, with decreases of 0.50 at 2 h, 0.30 at 4 h, and 0.15 at 6 h (Fig. 1b). For PKCε, the decreases were approximately 0.33 at 2 h, and ~0.20–0.23 for the remainder of the experiment (Fig. 1b). Removal of serum *per se* at this time point (2 h) had no effect on the expression levels of PKCα or PKCε (Fig. 1c). The relevance of both isoforms in neuronal systems is established by evidence that both are expressed in rat cortex (Fig. 1d). Other PKC isozymes (namely PKCβ and PKCζ,) were also monitored, but their levels were barely detectable and did not appear to change with the treatments tested. Tubulin expression was used as a control and also remained relatively unchanged throughout the experiments.

Morphological analysis of APP distribution following chronic PDBu treatment

As noted, regardless of whether cells were incubated in sf medium or c medium, PKCα was down-regulated following long-term exposure to phorbol esters. The effect on intracellular

APP distribution under these conditions was monitored using APP fused to GFP (Fig. 2). Cells were analyzed both qualitatively (Fig. 2a and b) and quantitatively (Fig. 2d). For quantitative analysis, 30 cells in triplicate were counted and scored for fluorescent TGN and cytoplasmic vesicular structures. Syntaxin 6 was used as a TGN marker in co-localization studies to confirm TGN scoring (Fig. 2c). Following long-term PDBu exposure, two significant qualitative effects were observed: an increase in the intensity and apparent volume of the fluorescent TGN structure and a dramatic decrease in the number of fluorescing cytoplasmic vesicles. The number of cells with fluorescent vesicles dropped drastically from 70% to 15% (Fig. 2d). Similar results were obtained regardless of whether cells were incubated in sf or c medium.

Co-localization of APP with PKC isozymes and organelle markers

Absence of APP–GFP incorporation into budding TGN vesicles following chronic exposure to PDBu was confirmed by confocal microscopy (Fig. 3). As expected, the PKCα immunoreactivity also decreased although syntaxin 6 immunoreactivity was maintained. Merged images confirmed that, under basal conditions, APP–GFP, PKCα, and syntaxin 6 were especially co-localized at the TGN (Fig. 3, white speckles). However, following chronic PDBu exposure, syntaxin 6 immunoreactive vesicles (red) were apparently devoid of APP–GFP fluorescence.

Contrary to the results observed for PKCα in sf medium, chronic exposure to PDBu led to increased PKCε immunoreactivity which co-localized in the juxta-plasma membrane region identified by clathrin immunoreactivity (red) (Fig. 4).

Acute PDBu application to cells chronically exposed to PDBu

Previous studies have demonstrated increases in sAPPα secretion following short-term exposure of cells to PDBu. This increase is likely to have several contributing factors, including enhanced vesicle budding from the TGN (Xu *et al.* 1995). Hence, cells labeled with [^{35}S]methionine were exposed to PDBu for increasing periods of time, and then subjected to a subsequent 1 h exposure to PDBu. The conditioned media were collected, immunoprecipitated with 6E10, and tested for sAPPα levels. A typical induction was evident when cells were not pre-incubated with PDBu. With only a 2 h pre-incubation, the response was already highly compromised (Fig. 5a). When pre-incubation periods with PDBu were increased to 4 h or more, subsequent acute stimulation with a spike of additional PDBu caused none of the typical stimulated release in sAPPα (Fig. 5a). Hence, the 4.5-fold increase in sAPPα for the APP751/770 isoform, evident with the 1 h PDBu spike in the absence of PDBu pre-incubation (Fig. 5a, 0 time point), was highly diminished by 4 h pre-incubation with PDBu. In summary, acute stimulation of the α-secretase pathway during long-term exposure to PDBu was considerably compromised. The failure of cells to respond by further increasing sAPP production correlated with down-regulation of PKCα (Fig. 1), and is consistent with the notion that PKCα in particular controls the α-secretase pathway, probably by modulating budding of APP transport vesicles at the TGN (Fig. 2).

Cumulative production of total sAPP following PKCα down-regulation

Although APP-regulated cleavage cannot be stimulated following PKCα down-regulation, sAPP is still being produced as is evident from Fig. 5b. Under the experimental conditions tested (−/+ PDBu), sAPPt (total sAPP) accumulated in the extracellular medium over time, albeit at different rates. For cells grown in sf medium, during the initial time points (2 and 4 h) continued PDBu exposure resulted in approximately three and four times more sAPP accumulating in the media, respectively (Fig. 5b and c), whereas in the absence of PDBu only a twofold increase was obtained. At these earlier time points, PKCα was not yet completely down-regulated for sf and c media, and thus PDBu still activated PKC, resulting

in the observed increases in sAPPt production. Interestingly, with continued PDBu exposure, the increased accumulation of sAPPt in the conditioned media per unit time levels off, with fold-increases reaching seven-to eightfold by 8–10 h, respectively (Fig. 5b and c). In the absence of PDBu, the rate of sAPPt production is maintained throughout the experiment, and the accumulated levels of sAPPt approach those obtained with PDBu, rising to fiveand sevenfold by 8 and 10 h (Fig. 5c). For cells maintained in c medium, the increases are more modest, resulting in a maximum fourfold sAPPt increase by 10 h. From the data presented above, it is evident that although PKCα is down-regulated and that the fluorescent signal from holo APP–GFP (hAPP) is no longer incorporated into visible vesicular structures, sAPP is still produced and secreted. Thus, the media collected were tested for both sAPPt and sAPPα (Fig. 5d). It appears that in sf medium sAPPt increases with time up to the 10 h time point, whereas sAPPα increases initially but remains constant from 6 to 10 h, suggesting that there is a tendency toward relatively more amyloidogenic processing as PKCα is down-regulated.

PKCα independent APP processing and Aβ production

To address amyloidogenic processing, COS-7 cells were labeled with [^{35}S]-methionine and incubated in sf and c media, with and without PDBu, and the medium was immunoprecipitated with 6E10. The resulting immunoprecipitates were analyzed for sAPPα (not shown) and Aβ (Fig. 6a). As discussed above, PDBu produced increases in sAPPα for cells maintained in sf and c media, although the increases were greater in sf medium. Enhanced sAPPα production is often accompanied by decreased Aβ production under acute exposure to PDBu. When the experiment was carried out in c medium, Aβ could not be detected; however, when sf medium was used, an increase in Aβ production was clearly visible (Fig. 6a). Hence, increased Aβ production occurred even when PKCα was down-regulated. Similar increases were obtained if cells were transfected with the Swedish APP mutant (Fig. 6b). However, given the unexpected concurrent increases in sAPPα and Aβ, levels of endogenous hAPP were also monitored (see below).

Given that increases in α and β cleavages for APP were both detected, intracellular levels of APP were monitored. Further, when experiments were carried out in sf medium, PKCε levels increased. Hence, cell lysates of COS cells cultured in sf or c medium and exposed to PDBu were subjected to immunoblot analysis. COS cells exposed to phorbol esters in sf medium showed a significant increase in intracellular levels of APP (Fig. 7a and b). Both the more abundant APP751/770 isoform and the less abundant APP695 isoform expressed in COS cells increased in a dose-dependent manner with time of exposure to PDBu. Maximal levels of APP expression were obtained with 1 μM PDBu for 8 h, when a greater than twofold increase could be detected (Fig. 7b). In contrast, when the experiment was carried out in c medium, no increases could be detected in the intracellular levels of hAPP (Fig. 7a). In this situation, PKCε is not up-regulated (Fig. 1). Thus, it seems reasonable to conclude that increased PKCε levels resulted in increased intracellular APP levels, perhaps because of a role for PKCε in APP transcription. This conclusion is also supported by a direct correlation between the concentration of PDBu added and increased intracellular levels of hAPP (Fig. 7b). Consequently, northern blot analysis was carried out on cells exposed to PDBu for long periods of time in sf (Fig. 7c) or c (data not shown) medium. The results showed that under conditions where PKCε was up-regulated, APP mRNA steady state levels were also increased.

Effect of PKCα down-regulation on Aβ production

Given that increased Aβ production resulted from long-term exposure to PDBu of cells incubated in sf medium, and that this was accompanied by increased intracellular levels of hAPP and PKCα down-regulation, we monitored whether the latter alone could contribute

to increased Aβ production. Thus, APP cDNA carrying the Swedish mutation was transiently transfected into COS cells and therefore not under the control of the endogenous APP promoter. The Swedish mutant shows an approximately 10-fold increase in catalytic efficiency for the APP : β-secretase reaction. Additionally, experiments were carried out at a total time of 4 h PDBu exposure (Fig. 6b). At this time, PKCα was considerably down-regulated, with only low levels of protein being detected (Fig. 1), and intracellular levels of endogenous APP were only marginally increased (Fig. 7a). Under these conditions, Aβ production was dramatically increased (Fig. 6b). Taken together, we conclude that our data are most consistent with the formulation that down-regulation of PKCα results in decreased TGN budding and a consequent retention of APP in the TGN, leading to increased Aβ production.

Discussion

Various reports have indicated that PKC plays important roles at several stages of APP processing, including sAPPα production, perhaps by controlling APP exit from the TGN. However, the phorbol ester sensitive molecular players involved in regulated APP shedding have remained elusive (Ikin *et al.* 2007). Up to now, most studies addressing regulated secretion have primarily employed acute PKC activation protocols. In our hands, both PKCα and PKCε were down-regulated by exposing to PDBu in c medium following long-term exposure of COS cells to phorbol esters. However, in sf medium, we observe up-regulation of PKCε and a strong down-regulation of PKCα. Elucidation of the molecular basis for the differential behavior of the PKC isozymes will require additional investigation.

In our hands, down-regulation of PKCα alone is sufficient to abolish the ability of additional PDBu to stimulate sAPPα production. However, with increasing periods of exposure to PDBu, sAPPα production plateaus for cells maintained in sf medium, while sAPPt continues to increase. In fact, direct measurement of Aβ shows that its production is dramatically increased as PKCα is down-regulated. Our results thus demonstrate roles for PKCα both in enhancing sAPPα generation and in diminishing amyloidogenic APP processing. This predicts the corollary that PKCα down-regulation almost completely inhibits stimulation of sAPPα production and leads to increased Aβ production (Fig. 6 and 7), and our data bear this out. Consistent with this role for PKCα, fibroblasts from Alzheimer patients exhibit down-regulation of PKCα and defective metabolism (Bergamaschi *et al.* 1995).

Mechanistically, PKCα down-regulation also affects the subcellular localization of APP, leading to its apparent accumulation in the TGN. Simultaneously, a dramatic decrease was observed in detectable APP-containing cytoplasmic vesicles, perhaps resulting from a diminution in budding of these vesicles at the TGN (Fig. 3) (Bergamaschi *et al.* 1995; Rossner *et al.* 2001), but we cannot formally exclude the possibility that these vesicles are endosomes that disappear because PKC activity is involved in endocytosis (Prevostel *et al.* 2000; Becker and Hannun 2003).

Most $A\beta^{1-40}$ and $A\beta^{1-42}$ peptides are believed to be generated in the TGN or in the endocytic pathway. Given that long-term exposure to phorbol esters appears to stimulate Aβ production to a greater extent than sAPPt secretion (Figs 5 and 6), one can conclude that PKCα down-regulation favors amyloidogenic APP processing. That is, increased Aβ production is not solely the result of increased APP mRNA expression, as discussed below, but is also a consequence of altered processing. Indeed, the results observed following transfection of an APP cDNA clearly illustrate this point (Fig. 6b).

Given the documented effects of PDBu on the AP-1 promoter which result in increased APP mRNA levels (Trejo *et al.* 1994), we addressed how long-term exposure to PDBu might affect APP transcription. A significant increase in the intracellular levels of hAPP was observed for cells maintained in sf medium (Fig. 7). Additionally, cells exposed to increasing concentrations of PDBu for increasing periods of time revealed a direct relationship between the PDBu concentration used and the observed increase in intracellular levels of hAPP. Thus, PDBu may be stimulating the APP promoter. Consistent with this model, both PKCα and PKCε have been reported to regulate transactivation of the AP-1 site in the APP promoter (Uberall *et al.* 1994). Further interplay between APP and PKCε expression are suggested in a recent study by Liron *et al.* (2007) in which they show that APP over-expression results in a specific and significant down-regulation of PKCε, with no change in the levels of other PKC isozymes.

In conclusion, although short-term phorbol ester treatment inhibits Aβ production, long-term exposure favors Aβ production. Consistent with previous reports (Jolly-Tornetta and Wolf 2000), PKCα appears to be involved in phorbol ester-regulated sAPPα secretion. However, PKCα down-regulation does not inhibit Aβ production. Further, the data herein support (but do not establish) the notion that PKCα down-regulation leads to failure of APP packaging into nascent vesicles budding from the TGN or failure of the budding process *per se*. Nonetheless, even in the absence of detectable APP-bearing vesicles, APP is processed, and high levels of sAPP and Aβ are still produced. PKCε, on the other hand, does not appear to be involved in regulating proteolytic cleavage of APP to an important degree, but rather appears to play a key role in modulating APP expression. Hence, PKCα and PKCε are differentially involved in APP metabolism. As discussed in the introduction, transfection with PKCα or PKCε isoforms leads to increased sAPP production (Kinouchi *et al.* 1995). However, given our data, it seems reasonable to assume that those increases have different origins. In the first case, increased sAPP secretion is likely because of enhanced APP metabolism stimulated by PKCα, whereas, in the case of PKCε, the increase is probably because of increased APP expression. These differential effects and time courses related to PKC isozyme biology must be taken into account when developing therapeutic drugs aimed at lowering Aβ generation by targeting PKC mediating signaling.

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Abbreviations used

ADAM	a disintegrin and metalloproteinase
AP-1	activator protein-1
APP	amyloid precursor protein
Aβ peptide	amyloid-β peptide
c medium	complete medium
COS	monkey kidney
DMEM	Dulbecco's modified Eagle's medium
GFP	green fluorescent protein

hAPP	holo APP
PDBu	phorbol-12,-14-di-butyrate
PKC	protein kinase C
sAPP	soluble APP
sAPPt	total sAPP
SDS	sodium dodecyl sulfate
sf medium	serum-free medium
TGN	*trans* Golgi network

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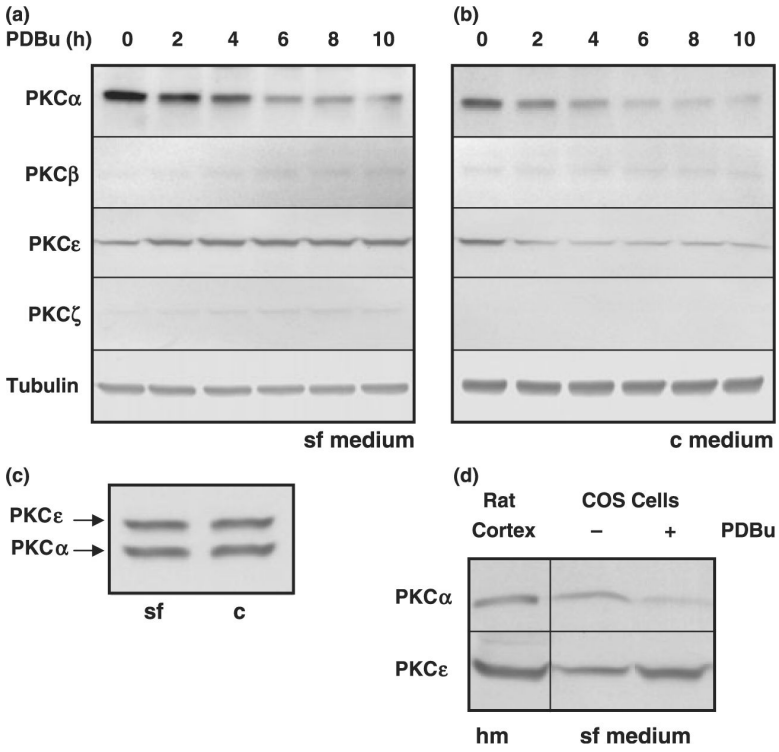

Fig. 1.

PKC isozyme expression profiling of COS cells. The expression of the main PKC isozymes was assessed in COS-7 cell lysates by immunoblotting with PKC isoform specific antibodies, following exposure to PDBu for the indicated periods (0, 2, 4, 6, 8, and 10 h). (a) Cells were incubated in serum-free medium (sf medium). (b) Cells were incubated in complete, serum containing, medium (c medium). Tubulin expression levels were monitored as a control. (c) The effect of removal of serum alone (− PDBu) for 2 h on the levels of PKCα and PKCε was monitored. Antibodies to both isoforms were used simultaneously. (d) Levels of PKCα and PKCε in rat cortex homogenates (hm) were compared with COS cells under basal conditions (−) and following 8 h exposure to PDBu (+).

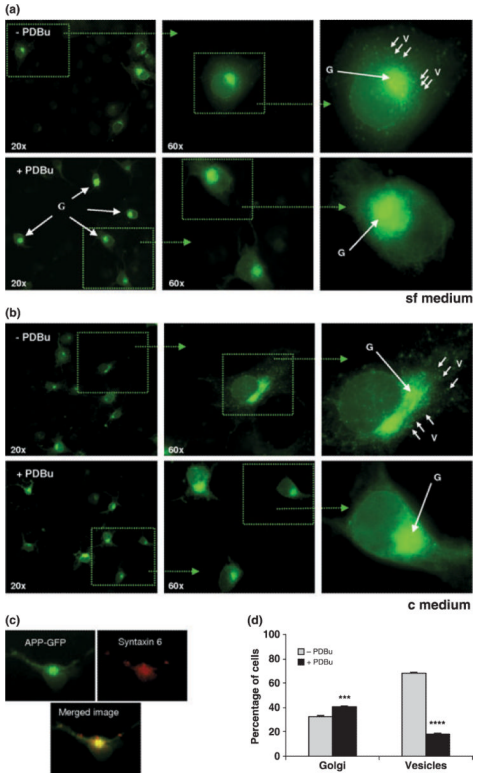

Fig. 2.

Intracellular localization of APP following down-regulation of PKCα. COS-7 cells were transfected with APP695–GFP, incubated for 8 h either in sf or c medium (both in the absence or presence of PDBu), and monitored for subcellular APP distribution. (a) Comparison of APP subcellular localization when cells were incubated in sf medium under control conditions and in the presence of PDBu. (b) Comparison of APP subcellular localization when cells were incubated in c medium under control conditions and in the presence of PDBu. (c) Co-localization of transfected APP695–GFP with the TGN/Golgi marker syntaxin 6. (d) Comparison of the number of cells exhibiting intensely fluorescent TGN or containing a large number of fluorescent cytoplasmic vesicles in sf medium following PDBu exposure. Data are expressed as mean ± SEM of triplicate determinations of three independent experiments. Statistical significance analysis was conducted by ANOVA followed by the two-tail Student's *t*-test. Statistical significance differences against control determinations (− PDBu) are presented as ***$p < 0.001$; ****$p < 0.0001$. G, Golgi; V, Vesicles.

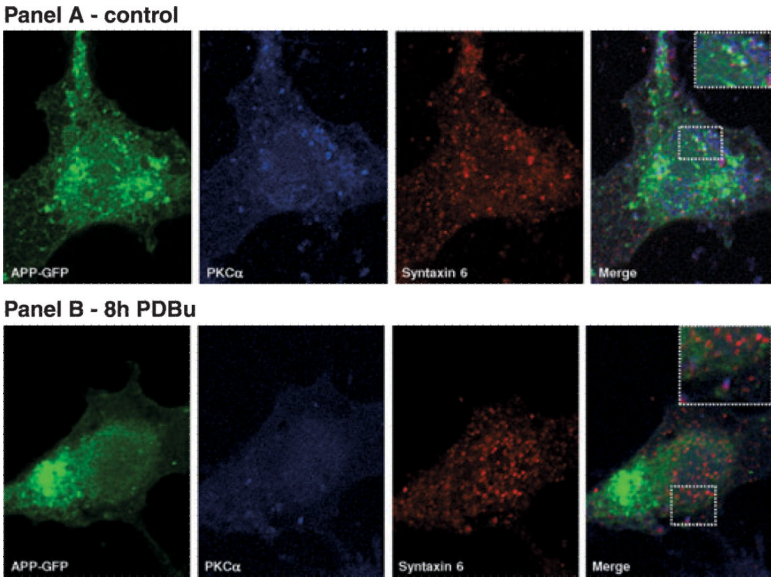

Fig. 3.

Co-localization of APP, PKCα and syntaxin 6. COS-7 cells transfected with APP695–GFP were analyzed for co-localization with PKCα and syntaxin 6. Observations were carried out under control conditions (panel a) and in the presence of PDBu for 8 h (panel b). PKCα was detected using a primary rabbit antibody (Gibco, BRL), and a secondary anti-rabbit Alexa Fluor 350 (Molecular Probes and BD Biosciences). Syntaxin 6 was detected using a primary mouse antibody, and a secondary anti-mouse Texas Red-conjugated antibody (Molecular Probes and BD Biosciences). Visualization was carried out with a Zeiss confocal microscope.

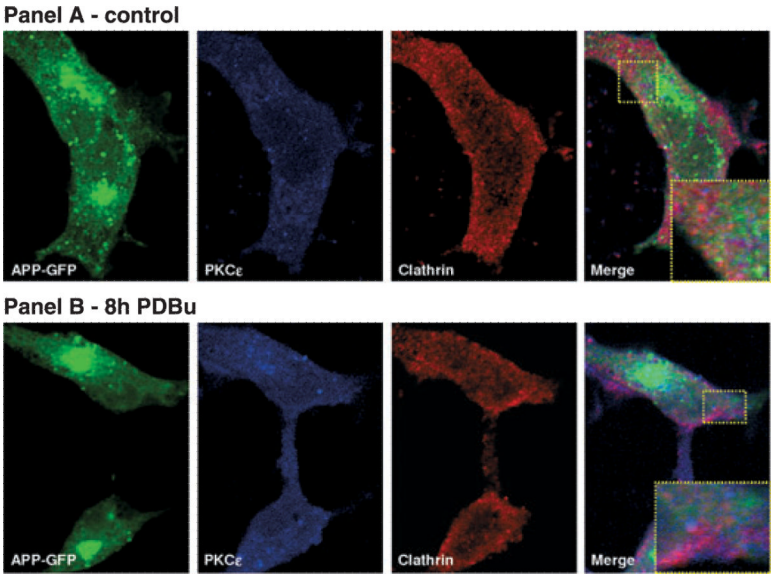

Fig. 4.

Co-localization of APP, PKCε, and clathrin. COS-7 cells transfected with APP695–GFP were analyzed for co-localization with PKCε and clathrin. Observations were carried out under control conditions (panel a) and in the presence of PDBu for 8 h (panel b). PKCε was detected using a primary rabbit antibody (Gibco, BRL), and a secondary anti-rabbit Alexa Fluor 350 (Molecular Probes and BD Biosciences). Clathrin (ICN Immunobiologicals) was detected using a primary goat antibody, and a secondary anti-goat Texas Red-conjugated antibody (Molecular Probes and BD Biosciences). Visualization was carried out with a Zeiss confocal microscope.

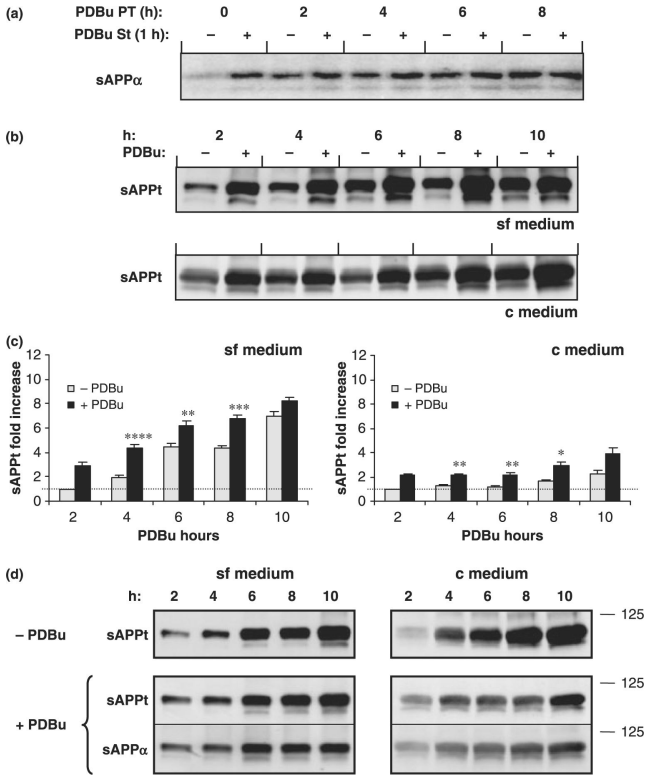

Fig. 5.

Effect of chronic PDBu exposure on sAPP secretion. (a) Regulated sAPP secretion. COS cells were subjected to a pre-treatment with PDBu (PDBu PT) from 0 to 8 h, followed by a 1 h exposure to PDBu stimulation (PDBu St), using serum-free medium. The 1 h medium from pulse chased cells was collected, and secreted sAPPα was immunoprecipitated with the antibody 6E10. Immunoprecipitates were separated by SDS–polyacrylamide gel electrophoresis and sAPPα was detected by autoradiography. (b) Cumulative sAPP secretion. Immunoblot analysis of sAPP accumulating in the extracellular milieu at 2 h intervals. COS-7 cells maintained in sf medium or in c medium were exposed to PDBu. The conditioned medium was separated on SDS–polyacrylamide gel electrophoresis and immunoblotted with the antibody 22C11. Total sAPP (sAPPt) is indicated. (c) Quantitative representation in fold increase of sAPPt produced in the absence or presence of PDBu, for cells maintained in sf or c medium. Data are expressed as mean ± SEM of triplicate determinations of three independent experiments. Statistical significance analysis was conducted by ANOVA followed by the two-tail Student's *t*-test. Statistical significance differences against control determinations (− PDBu) are presented as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. (d) Comparative immunoblot analysis of sAPPt and sAPPα produced by cells when exposed to PDBu medium in sf or c medium.

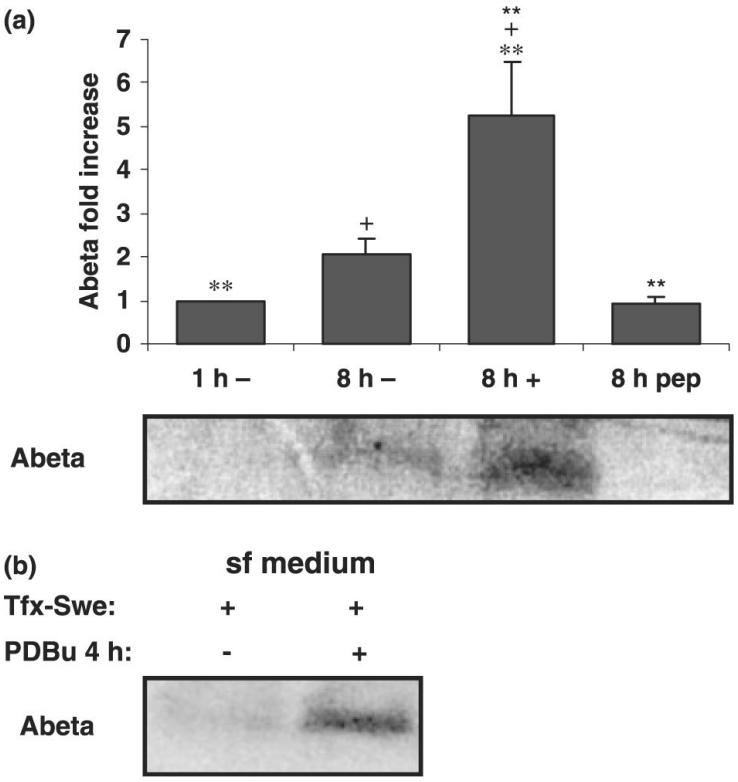

Fig. 6.

Effect of long-term PDBu exposure on Aβ production. (a) Metabolic labeling experiments were carried out in COS cells maintained in sf or c medium in the absence or presence of PDBu. Media from 8 h were collected, immunoprecipitated with antibody 6E10, and subjected to SDS–polyacrylamide gel electrophoresis analysis. Antibody specificity was tested by including the Aβ peptide for a control immunoprecipitation. Detected Aβ is indicated and quantified. Data are expressed as mean ± SEM of triplicate determinations from at least three independent experiments. Statistical significance analysis was conducted by ANOVA followed by the Tukey test. Statistical significance symbols used for statistically significant differences were $^{+}p < 0.05$ (between 8 h minus and plus PDBu determinations); $^{**}p < 0.01$ (between 1 h minus and 8 h plus PDBu determinations); $^{**}p < 0.01$ (between 8 h plus PDBu determinations, with or without Aβ peptide competition). (b) The procedure was repeated for cells transfected with APP^{Swe} cDNA (Tfx-Swe). PDBu exposure times were reduced to 4 h since at this time point APP expression levels remain largely unaltered.

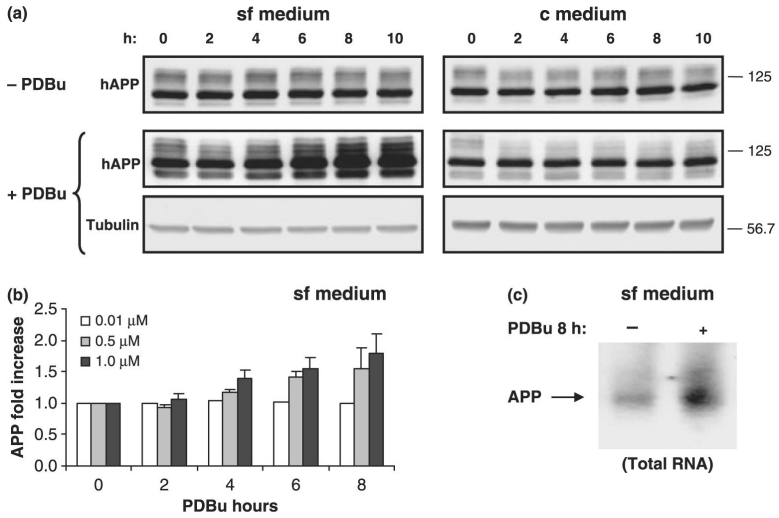

Fig. 7.

Effect of PDBu exposure on expression levels of APP. (a) Immunoblot analysis of holoAPP (hAPP) expression levels in COS-7 cells, using mouse monoclonal antibody 22C11. Cells were grown in the absence or presence of PDBu for up to 10 h without serum (sf medium) and with serum (c medium). Tubulin expression levels were monitored as a control. (b) PDBu dose effect on hAPP expression levels. Data are expressed as mean ± SEM. (c) Northern blot analysis of APP mRNA levels following long-term exposure to PDBu (8 h).