Regulated Formation of Golgi Secretory Vesicles Containing Alzheimer β-Amyloid Precursor Protein*

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Phorbol esters, activators of protein kinase C (PKC), regulate the relative utilization of alternative processing pathways for the Alzheimer β-amyloid precursor protein (β-APP) in intact cells, increasing the production of nonamyloidogenic soluble β-APP (sβ-APP) and decreasing that of neurototoxic β-amyloid (Aβ) peptide. The cellular and cellular bases of PKC-regulated β-APP cleavage are poorly understood. Here we demonstrate in a reconstituted cell-free system that activation of endogenous PKC increases formation from the trans-Golgi network of secretory vesicles containing β-APP and that this effect can be mimicked by purified PKC. The results demonstrate directly that PKC is involved in regulation of secretory vesicle formation and provide a mechanism by which PKC may reduce the formation of the Aβ peptide characteristic of Alzheimer disease.

Alzheimer disease (AD) is a common neurodegenerative disorder characterized by dementia and by accumulation in the brain of extracellular deposits composed of β-amyloid peptide (Aβ) (Glenner and Wong, 1984). Signal transduction via protein phosphorylation governs the relative utilization of competing pathways for the metabolism of the β-amyloid precursor protein (β-APP) (Kang et al., 1987), the type I integral glycoprotein from which Aβ is derived by proteolytic cleavage. Activated protein kinase C (PKC) stimulates nonamyloidogenic β-APP cleavage (Buxbaum et al., 1990; Caporaso et al., 1992), generating soluble β-APP (sβ-APP) at the expense of other pathways, including that involved in the formation of Aβ (Hung et al., 1993). PKC-regulated β-APP cleavage does not require changes in the phosphorylation state of the β-APP cytoplasmic tail (da Cruz e Silva et al., 1993), suggesting that one or more molecules of the β-APP trafficking and processing apparatus are PKC substrate phosphoproteins involved in the mechanism by which PKC regulates β-APP cleavage.

Most β-APP resides intracellularly, codistributing with TGN38, a marker of the trans-Golgi network (TGN) (Caporaso et al., 1994). Thus, it seemed possible that PKC might exert its actions on regulated β-APP cleavage by redistributing β-APP out of its usual residence in the TGN and toward post-TGN compartments where it can undergo processing. This possibility is supported by studies demonstrating stimulation by phorbol esters of the release of glycosaminoglycans, intraluminal molecules of the constitutive secretory pathway (DeMaggio et al., 1993; Ohashi and Huttner, 1994). Therefore, we have tested the possibility that an important component of regulated β-APP cleavage is PKC-stimulated formation from TGN of constitutive secretory vesicles containing and transporting mature β-APP.

MATERIALS AND METHODS

Intact Cell Studies—Three plates of PC12 cells (10-cm dishes, 5 × 10^7 cells/dish) were labeled with 2 mCi/ml [35S]sulfate (Amersham Corp.) for 10 min and chased at 37°C in presence of fetal bovine serum/Life Technologies, Inc. (LC Services, Woburn, MA) for 15, 25, or 45 min. At the end of the incubation, media were collected and immunoprecipitated with β-APP amino-terminal antibody 22C11 (Caporaso et al., 1992). Cells were homogenized, and Golgi-rich membrane fractions were prepared by flotation using a stepwise sucrose gradient (Xu and Shields, 1993). The Golgi-rich fractions were immunoprecipitated with β-APP carboxy-terminal antibody 369 (Buxbaum et al., 1990). Immunoprecipitates were resuspended in 1% SDS and adjusted to equal amounts of total protein (Bradford), followed by 4–12% SDS-polyacrylamide gel electrophoresis analysis and autoradiography on Kodak X-Omat™ AR5 film.

Cefi-Free Vesicle Budding Assay—Confluent PC12 cells (5 × 10^6) were pulse-labeled with 2 mCi/ml [35S]sulfate (Amersham Corp.) for 5 min in sulfate-free medium (Life Technologies, Inc.) at 37°C. Cells from each 10-cm dish were homogenized using a stainless steel ball bearing homogenizer (18-m clearance) in 5 volumes of homogenization buffer (0.25 M sucrose, 1 mM magnesium acetate, 0.5 mM EDTA, 0.2 mM CaCl₂, and proteinase inhibitors) (Xu and Shields, 1993). A postnuclear supernatant was prepared (Tooze and Huttner, 1992) and centrifuged (Beckman TJ-25 rotor) at 14,000 × g for 10 min at 4°C. The pellet was then washed and resuspended in 300 μl of homogenization buffer. Aliquots (100 μl of membrane preparation) of resulting suspension, the “TGN-rich fraction,” were incubated in a final volume of 250 μl at 37°C for 30 min in the presence of an energy-regenerating system containing 1 mM ATP and 0.2 mM GTP in the absence or presence of 1 mg of cytosol protein/ml (Xu and Shields, 1993). At the end of the incubation period, samples were centrifuged (Beckman TLA-45 rotor) at 14,000 × g for 10 min at 4°C. The pellets and supernatants were separated and immunoprecipitated with antibody 369 and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The redistribution of 35S-sulfated β-APP into the supernatant provides a measure of budding of vesicles containing β-APP. Treatment of the vesicle-containing supernatant with proteinase K (25 μg/ml) (Boehringer Mannheim) resulted in consistent with the predicted existence of the β-APP ectodomain within a vesicular lumen (data not shown).

Quantification and pairwise analyses were carried out using a Bio-Rad phosphor imaging system (Molecular Analyst™ version 2.0 software). For each experiment, the level of budding observed in the presence of an aliquot of a standard preparation of cytosol was taken as 1 arbitrary unit of budding efficiency, and other levels within a given
Regulated Budding of β-APP-containing Vesicles

RESULTS AND DISCUSSION

Golgi-rich Fractions from Phorbol Ester-treated Cells Are Depleted of β-APP—In intact cells, PDBu caused a depletion of Golgi-associated mature, sulfated β-APP (Fig. 1). Sulfated β-APP holoprotein content of the Golgi-rich fraction (Xu and Shields, 1993) from metabolically labeled cells chased in the presence of PDBu for 5 or 15 min was approximately 30 and 40%, respectively, of that observed in the absence of PDBu (Fig. 1, left panel). Concomitantly, appearance of sβ-APP in the medium was enhanced severalfold at all times studied (Fig. 1, right panel).

PKC Activation Stimulates TGN Vesicle Formation in a Cell-free System—Regulation by protein phosphorylation of the biogenesis of β-APP-containing vesicles was directly demonstrated using a modification of an in vitro assay of secretory vesicle formation from a TGN-rich fraction (Ohashi and Huttner, 1994). This assay allows study of the effects of cell-impermeant agents such as PKC and GTPγS. Since it is well established that several cytosolic molecules play important roles in vesicle budding (for review, see Rothman (1994)), budding from isolated TGN was tested in the absence and presence of cytosol.

Cytosol stimulated the formation of nascent vesicles containing β-APP (Fig. 2, autoradiogram, lane 1 versus lane 6; Fig. 3A, lane 1 versus lane 2). This stimulatory effect was mimicked by purified PKC (Woodgett and Hunter, 1987), with a half-maximal effect at 9 μg/ml and a maximal effect at 25 μg/ml (Fig. 2, graph; Fig. 3A, lane 1 versus lane 6). In the presence of cytosol, the addition of PDBu increased vesicle budding, presumably due to activation of endogenous PKC (Fig. 3A, lane 2 versus lane 5). The rate of vesicle budding observed in the presence of cytosol alone or of cytosol plus PDBu/PKC was greatly reduced in the presence of PKC inhibitor peptide-(19–36) (House and Kemp, 1987), with a half-maximal effect at 20 μM AlF4⁻ (Fig. 3A, lane 2 versus lane 6). Means ± S.E. for three experiments are shown. asterisk, different from cytosol alone (p < 0.01); dagger, different from PDBu/PKC alone (p < 0.01).

Modulation of Vesicle Budding in the Cell-free Assay—In some experiments, modulation of vesicle budding was tested using (together and separately) purified rat brain PKC (Woodgett and Hunter, 1987) and/or PKC inhibitor peptide-(19–36) (House and Kemp, 1987). PKC inhibitor peptide-(19–36), used at 200 μM as recommended (House and Kemp, 1987), was synthesized at the Kek Foundation Protein Synthesis and Sequencing Facility at Yale University, New Haven, CT. Effects on vesicle budding were also tested for GTPγS (30 μM) (Boehringer Mannheim) or (AlF4)⁻ (80 μM AlF4⁻ plus 6 mM F⁻).

Stripping of Golgi-rich Membranes Prior to Use in the Cell-free Vesicle Budding Assay—In some experiments, TGN-rich fractions derived from [35S]sulfate-labeled cells were washed with low (control) or high salt and analyzed for vesicle budding in the absence or presence of PDBu (1 μM)/purified PKC (25 μg/ml) or cytosol (1 mg/ml). For washing of membrane preparations, the [35S]sulfate-labeled TGN-rich fractions were adjusted to 100 or 400 mM potassium acetate and incubated at 4°C for 10 min followed by 10 min of centrifugation (Beckman TLA-45 rotor) at 14,000 × g at 4°C. The pellets were then washed in homogenization buffer, resuspended in buffer of the same composition, and used in the cell-free vesicle budding assay.

Fig. 1. PKC activation in intact PC12 cells leads to redistribution of holo-β-APP from a Golgi-rich fraction to sβ-APP in conditioned culture medium. Cells were metabolically labeled with [35S]sulfate, followed by analysis of β-APP in a Golgi-rich membrane fraction from the cells and of sβ-APP in the culture medium. The autoradiograms are from one of two experiments, which yielded virtually identical results.

Fig. 2. Stimulation of vesicle budding from TGN by cytosol and by purified PKC. A TGN-rich fraction derived from [35S]sulfate-labeled cells was incubated under standard conditions in the presence of cytosol or of various concentrations of purified PKC followed by analysis of vesicle budding (see "Materials and Methods").

Table 1. Regulation of vesicle budding from TGN. A, effect of cytosol (1 mg/ml), PDBu (1 μM), PKC (25 μg/ml), and PKC inhibitor peptide-(19–36) (200 μM) on vesicle budding. B, reduction of vesicle budding by removal of energy regenerating system (–ERS), incubation at 20°C, or addition of either GTPγS (30 μM) or (AlF4)⁻ (80 μM AlF4⁻ plus 6 mM F⁻). Means ± S.E. for three experiments are shown. asterisk, different from cytosol alone (p < 0.01); dagger, different from PDBu/PKC alone (p < 0.01).
Kemp, 1987) (Fig. 3A, lane 2 versus lane 3, lane 7 versus lane 8). Cytosol caused a significant increase in vesicle budding from the TGN even in the presence of an optimally effective amount of PDBu/PKC (Fig. 3A, lane 6 versus lane 7), suggesting the presence of a cytosolic factor(s) in addition to PKC. A similar effect of cytosol was observed when cytosol was added to stripped Golgi membranes (see below) in the presence of a cytosolic factor(s) in addition to PKC. A similar additional stimulatory effect of cytosol was observed when cytosol was added to stripped Golgi membranes (not shown). The effect of cytosol alone was abolished in the absence of an energy-regenerating system or when incubation was carried out at 20°C, a temperature that blocks TGN exit (Fig. 3B).

GTPγS and Aluminum Fluoride Inhibit TGN Vesicle Formation in a Cell-free System—Vesicle budding is known to be dynamically regulated (see Bauerfeind and Huttner, 1993) for review). Numerous reports have implicated GTP binding proteins (both the small Ras-like class and the heterotrimeric class) in many processes of intracellular vesicular transport including formation of secretory vesicles from the TGN. (AlF4)3−, an activator of both stimulatory and inhibitory trimeric G proteins, diminished the budding of secretory vesicles containing β-APP, indicating the involvement of heterotrimeric G proteins. Consistent with this interpretation, GTPγS, a non-hydrolyzable GTP analogue, also reduced budding (Fig. 3B).

Activated Purified PKC Stimulates Vesicle Budding from Stripped Golgi Membranes, Suggesting That Budding Is Regulated by a Tightly Associated or Integral TGN Phosphoprotein—In order to investigate whether the PKC substrate that regulated budding was likely to be cytosolic or TGN-associated, the [35S]sulfate-labeled, TGN-rich fraction was washed with 100 mm (control) or 400 mm (high salt) potassium acetate. Control and high salt-washed TGN were then assayed for vesicle formation in the absence or presence of purified PKC or cytosol (Fig. 4). The ability of PKC to stimulate vesicle budding in the absence of cytosol was largely retained after stripping of the TGN with high salt. The results suggest that at least one important PKC target is likely to be a tightly associated cytosolic or integral TGN phosphoprotein, presumably acting downstream from various known components of the budding apparatus (Rothman, 1994).

Conclusions—The present results demonstrate that when appropriate PKC-mediated signal transduction systems are activated, β-APP is redistributed from the TGN to other cellular locations where it can encounter its processing enzymes, e.g. to the plasma membrane, where it is cleaved by “a-secretase,” another candidate PKC target (Bosenberg et al., 1993; Arribas and Massague, 1995). The data also support the idea that the so-called “constitutive” secretory pathway is subjected to important regulatory influences and that some of this regulation occurs via protein phosphorylation/dephosphorylation (De Matteis et al., 1993; Ohashi and Huttner, 1994). Our results indicate that PKC constitutes an important component of the contribution of cytosol to the regulation of budding. Moreover, the evidence suggests that at least one important substrate is tightly associated with the TGN.

In Alzheimer patients of the Swedish familial AD type, there is an abnormally low ratio of processing of β-APP via the non-amyloidogenic sβ-APP pathway relative to the amyloidogenic Aβ pathway (Felsenstein et al., 1994a). This ratio is normalized by activation of PKC, which enhances processing via the non-amyloidogenic pathway while decreasing processing via the amyloidogenic pathway (Felsenstein et al., 1994b; Citron et al., 1994). The present study reveals one cellular mechanism by which PKC produces these effects. In addition, the recent discovery that a major familial AD gene (Sherrington et al., 1995) encodes a protein homologous to the Caenorhabditis elegans sperm molecule spe4, which plays a role in membrane protein sorting (L. Hersh and Arduengo, 1992), raises the possibility that misorting of β-APP may contribute to some forms of AD. A more complete understanding of the molecules that control β-APP trafficking and processing events, as well as an understanding of how these molecules are regulated, should lead to new insights into the etiology, pathogenesis, and therapy of AD.

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Note Added in Proof—After this study was finished, additional evidence supporting the existence of a role for PKC in the regulation of TGN vesicle formation was described by Buccione et al. (R. Buccione, S. Bannykh, I. Santone, M. Baldassarre, F. Facchiano, Y. Bozzi, G. Di Camillo, A. Mironov, A. Luini, and M. De Matteis, submitted for publication) as well as by Simon et al. (Simon, J.-P., Ivanov, I. E., Shopsin, B., Hersh, D., Aadenik, M., and Sabatini, D. D. (1995) Cold Spring Harbor Symp. Quant. Biol. 60, 222).

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