Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4081–4084, April 1996
Cell Biology

Metabolism of Alzheimer β-amyloid precursor protein: Regulation by protein kinase A in intact cells and in a cell-free system

(protein processing/protein trafficking/protein phosphorylation)

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Contributed by Paul Greengard, December 28, 1995

ABSTRACT **Various compounds that affect signal transduction regulate the relative utilization of alternative processing pathways for the β-amyloid precursor protein (βAPP) in intact cells, increasing the production of nonamyloidogenic soluble βAPP (sβAPP) and decreasing that of amyloidogenic β-amyloid peptide. In a recent study directed toward elucidating the mechanisms underlying phorbol ester-stimulated sβAPP secretion from cells, it was demonstrated that protein kinase C increases the formation from the *trans*-Golgi network (TGN) of βAPP-containing secretory vesicles. Here we present evidence that forskolin increases sβAPP production from intact PC12 cells, and protein kinase A stimulates formation from the TGN of βAPP-containing vesicles. Although protein kinase A and protein kinase C converge at the level of formation from the TGN of βAPP-containing vesicles, additional evidence indicates that the regulatory mechanisms involved are distinct.**

Alzheimer disease (AD) is characterized by an intracranial amyloidosis that develops in an age-dependent manner. This amyloidosis appears to be dependent upon the production of β-amyloid peptide (Aβ) by proteolysis of its integral membrane precursor, the Alzheimer β-amyloid precursor protein (βAPP). Metabolism of the transmembrane βAPP occurs via various alternative trafficking and processing pathways (for review, see refs. 1 and 2). The "α-secretase" pathway involves transport of βAPP holoprotein to the cell surface where it is cleaved within the amyloidogenic Aβ domain to produce a large amino-terminal fragment [soluble βAPP (sβAPP)], which is released from the cell (3–5). The resultant C-terminal fragment is retained by the cell and transported via clathrin-coated vesicles to lysosomes for degradation (6, 7). By cleaving βAPP molecules within the Aβ domain, the α-secretase pathway prevents Aβ production. The "β-secretase" pathway (8) specifies cleavage at the amino terminus of the Aβ domain of βAPP, yielding a potentially amyloidogenic C-terminal fragment. This and other potentially amyloidogenic fragments are then presumably further cleaved by an activity designated "γ-secretase" to yield the Aβ peptide, which is released into culture medium and body fluids (9–11).

Signal transduction via protein phosphorylation governs the relative utilization of competing pathways for the metabolism of βAPP. Activated protein kinase C (PKC) stimulates nonamyloidogenic βAPP cleavage, generating sβAPP at the expense of other pathways, including that involved in the formation of Aβ (12–15). PKC-regulated βAPP cleavage does not require changes in phosphorylation state of the βAPP cytoplasmic tail (16, 17). This suggests that the mechanism by which PKC regulates βAPP cleavage involves phosphorylation of one or more molecules of the βAPP trafficking and processing apparatus.

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Recently we have reconstituted the formation of βAPP-containing vesicles from the *trans*-Golgi network (TGN) in a cell-free system and demonstrated that PKC redistributes βAPP out of its usual residence in the TGN and toward post-TGN compartments where it can undergo processing (18). Other studies (19) indicated that protein kinase A (PKA) activating reagents, like PKC-activating reagents, could stimulate the release of constitutive secretory proteins from the TGN via a mechanism involving enhanced formation of post-TGN transport vesicles. Here we demonstrate that PKA-activating reagents increase sβAPP secretion from intact rat pheochromocytoma (PC12) cells and that purified PKA stimulates formation of βAPP-containing secretory vesicles from the TGN in a cell-free vesicle budding system. As with the putative PKC substrates (18), at least one PKA substrate is likely to be a TGN-integral phosphoprotein or a cytosolic phosphoprotein tightly associated with the TGN. Moreover, our results indicate that PKA and PKC exert their actions on vesicle budding from the TGN by distinct mechanisms.

MATERIALS AND METHODS

Intact Cell Studies. PC12 cells (10^7 cells per 60-mm dish) were labeled with 500 μCi/ml of [^{35}S]methionine (DuPont/NEN) for 10 min, chased at 20°C for 2 hr, and then incubated at 37°C for various times, as indicated, in the presence or absence of various concentrations of forskolin or 1 μM PDBu (LC Services, Woburn, MA). At the end of the incubation period, media were collected and immunoprecipitated with antibody 22C11 (14). Cell lysates were prepared (20) and immunoprecipitated with antibody 369. Samples were analyzed by 4–12% SDS/PAGE analysis and autoradiography on Kodak X-Omat AR5 film. Quantification and pairwise analyses were carried out using a Bio-Rad PhosphorImager (MOLECULAR ANALYST version 2.0 software).

Cell-Free Vesicle Budding Assay. Confluent PC12 cells (5×10^7) were pulse-labeled with 2 mCi/ml [^{35}S]sulfate (Amersham) for 5 min in sulfate-free medium (GIBCO/BRL) at 37°C. Cells from each 10-cm dish were homogenized using a stainless steel ball bearing homogenizer (18 μm clearance) in 1 ml of homogenization buffer (0.25 M sucrose/10 mM HEPES, pH 7.2/1 mM magnesium acetate/0.5 mM EDTA/0.2 mM $CaCl_2$/proteinase inhibitors; ref. 20). A postnuclear supernatant (PNS) was prepared (20) and centrifuged (Beckman model TLA-45 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

Abbreviations: TGN, *trans*-Golgi network; βAPP, Alzheimer β-amyloid precursor protein; Aβ, β-amyloid peptide; sβAPP, soluble βAPP; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PKA, protein kinase A (cAMP-dependent protein kinase); PKC, protein kinase C; OA, okadaic acid; AD, Alzheimer disease.

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presence of an energy regenerating system containing 1 mM ATP and 0.2 mM GTP in the absence or presence of cytosol (1 mg of protein per ml) (20). At the end of the incubation period, samples were centrifuged (Beckman model 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/PAGE and autoradiography. The redistribution of [^{35}S]sulfate-labeled βAPP into the supernatant provides a measure of budding of vesicles containing βAPP. Quantification and pairwise analyses were carried out using a Bio-Rad PhosphorImager (MOLECULAR ANALYST version 2.0 software). In all experiments, the level of budding observed in the presence of cytosol alone was taken as 1 arbitrary unit of budding efficiency, and other levels within a given experiment were normalized to this value. Treatment of the vesicle-containing supernatant with proteinase K (25 μg/ml; Boehringer Mannheim) resulted in digestion of the βAPP-like 22C11 (ectodomain)-immunoreactive material in the presence, but not in the absence, of 1% Triton X-100, consistent with the predicted existence of the βAPP ectodomain within a vesicular lumen (data not shown).

Modulation of Vesicle Budding in the Cell-Free Assay. Modulation of vesicle budding was tested using purified catalytic subunit of PKA (21), purified rat brain PKC (22), PKA inhibitor peptide, [5–24]amide (23) and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (30 μM) (Boehringer Mannheim), as indicated. PKA inhibitor peptide, used at 20 μM, was synthesized at the Keck Foundation Protein Synthesis and Sequencing Facility at Yale University (New Haven, CT).

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

RESULTS AND DISCUSSION

Forskolin Stimulates sβAPP Release from Intact PC12 Cells. In order to evaluate the possible effect of PKA activation on secretion of the α-secretase product, sβAPP, from intact cells, we utilized forskolin to increase intracellular cAMP levels. PC12 cells were labeled for 10 min with [^{35}S]methionine followed by a 2-hr chase in complete medium at 20°C. At this temperature, secretory proteins accumulate in the TGN (20, 24, 25). After a 2-hr block at 20°C, cells were incubated at 37°C in the absence or presence of forskolin for up to 120 min. Cell lysates (data not shown) and media (Fig. 1) were immunoprecipitated with antibodies recognizing the cytoplasmic tail (369) and the amino-terminal domain (22C11) of βAPP, respectively. The effect of forskolin on sβAPP production was dose dependent with a maximal stimulatory effect of approximately 3-fold at 3 μM forskolin 1,9-Dideoxyforskolin, an analogue of forskolin that does not activate adenylyl cyclase, failed to stimulate sβAPP secretion at the concentrations tested (1–10 μM; data not shown). Forskolin exhibited a profile for sβAPP release, which was similar to that of phorbol ester, with approximately 45% of total labeled APP released after a 90-min chase in the presence of forskolin as compared with 12% released from control untreated cells (Fig. 2). These data indicate that the α-secretase pathway can be activated by increasing intracellular cAMP. Similar results have recently been reported using a murine microglial cell line (26).

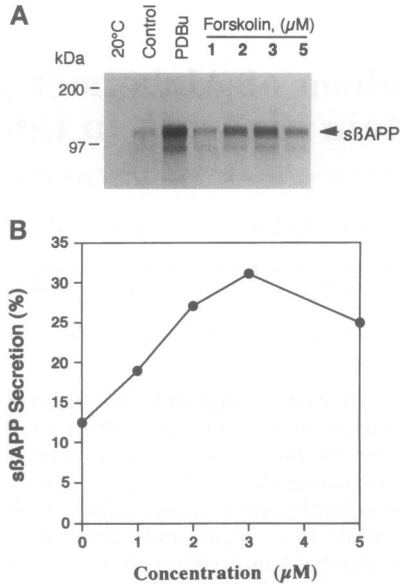

FIG. 1. Forskolin stimulates sβAPP secretion from intact PC12 cells. PC12 cells were pulse-labeled with 500 μCi/ml [^{35}S]methionine for 10 min and chased for 2 hr at 20°C. A portion of the 20°C chase medium was collected, and cells were further incubated at 37°C for 60 min in the absence (Control) or presence of 1 μM PDBu or the indicated concentrations of forskolin. Samples of the culture medium (*A*) or cell lysate (data not shown) were immunoprecipitated with antibodies 22C11 or 369, respectively, followed by analysis on 4–12% SDS/PAGE. Arrow indicates the position of sβAPP. sβAPP secretion (%) (*B*) was calculated as [sβAPP/(βAPP + sβAPP)] × 100%.

PKA Stimulates *in Vitro* Formation from the TGN of βAPP-Containing Vesicles. Recently, regulation by PKC and G proteins of the biogenesis of βAPP-containing vesicles was demonstrated using a modification of an *in vitro* assay of secretory vesicle formation from a TGN-rich fraction (18). In that study, *in vitro* budding from isolated TGN was stimulated by cytosol, and purified PKC was able to support budding of βAPP-containing vesicles from high salt washed TGN-rich membrane fractions even in the absence of cytosol. Therefore, in the present investigation, *in vitro* budding from isolated TGN was tested in both the absence and presence of cytosol.

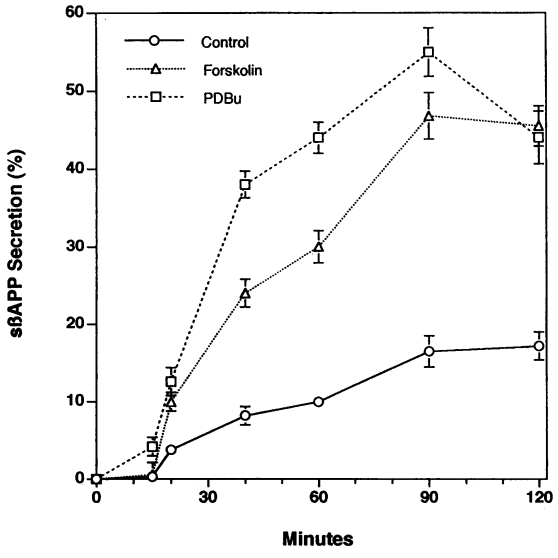

FIG. 2. Kinetics of forskolin-stimulated sβAPP secretion. PC12 cells were pulse labeled with [^{35}S]methionine for 10 min and chased for 2 hr at 20°C. Cells were then incubated at 37°C for the indicated times in the absence or presence of 3 μM forskolin or 1 μM PDBu, followed by analysis as described in the legend to Fig. 1.

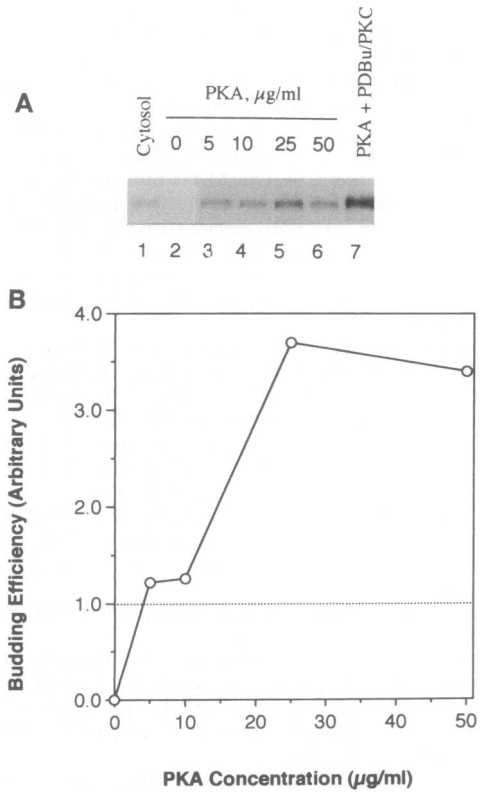

FIG. 3. Activation of vesicle budding from TGN by cytosol and by purified PKA. (*A*) A TGN-rich fraction derived from [^{35}S]sulfate-labeled PC12 cells was incubated under standard conditions in the presence of cytosol (1 mg of protein per ml), various concentrations of purified PKA catalytic subunit, or 25 μM purified PKA catalytic subunit, 1 μM PDBu, and 25 μM PKC, followed by analysis of vesicle budding. (*B*) Quantitation of vesicle budding. The level of budding observed in the presence of cytosol alone was taken as 1 arbitrary unit of budding efficiency, and other levels within a given experiment were normalized to this value.

Cytosol stimulated the formation of nascent vesicles containing βAPP (Fig. 3; Fig. 4, lane 1 vs. 6; $P < 0.005$). This stimulatory effect was mimicked by purified catalytic subunit of PKA, with a half-maximal effect at 10–15 μg/ml and a maximal effect at 25 μg/ml (Fig. 3). OA, an inhibitor of protein phosphatases 1 and 2A, stimulated budding by about 2-fold in the presence of cytosol (Fig. 4, lane 6 vs. 7; $P < 0.01$). In the presence of cytosol, βAPP-containing vesicle budding was enhanced by about 1.5-fold by purified catalytic subunit of PKA (Fig. 4, lane 6 vs. 8; $P < 0.02$). The combination of PKA plus OA in the reaction mixture caused a synergistic effect on vesicle budding (Fig. 4, lanes 6–9). The enhanced level of budding observed in the presence of PKA alone, OA alone, or PKA plus OA, was abolished by a PKA inhibitor peptide (data not shown).

A Tightly Associated or Integral TGN Phosphoprotein May Be an Important PKA Target That Regulates Vesicle Budding. Rothman and colleagues (27, 28) have identified several cytosolic factors required for vesicle budding and fusion during intra-Golgi transport. In addition, in many cell-free systems, cytosolic extracts are necessary to support vesicle budding (29), exocytosis (30, 31), and endocytosis (27). Indeed, in our system, cytosol was able to elevate further the level of vesicle budding beyond that observed in the presence of optimal concentrations of PKA plus PKC, indicating the involvement of a cytosolic factor in the budding process (Fig. 4, lane 5 vs. 10; $P < 0.02$).

In a previous study, evidence was presented that PKC-stimulated vesicle budding involves a tightly associated or

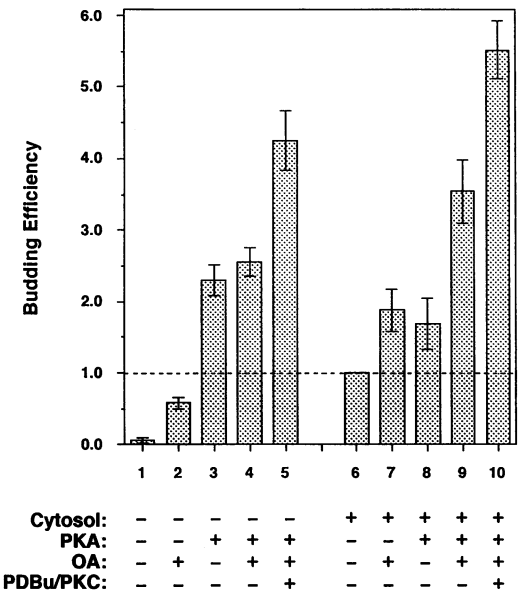

FIG. 4. Regulation of vesicle budding from TGN. A TGN-rich fraction derived from [^{35}S]sulfate-labeled PC12 cells was incubated under various conditions as indicated. Cytosol (1 mg of protein per ml), purified PKA catalytic subunit (25 μM), okadaic acid (OA) (1 μM), PDBu (1 μM), and purified PKC (25 μM) were present as indicated. Data represent mean ± SEM for three experiments.

integral TGN phosphoprotein (18). In the present study, we found that stimulation of vesicle budding by PKA also involved a TGN-associated substrate. Thus, the [^{35}S]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 catalytic subunit of PKA, cytosol, or both (Fig. 5). The ability of PKA/OA (Fig. 5) or of PKA alone (data not shown) to stimulate vesicle budding in the absence of cytosol was largely retained after stripping of the TGN with high salt.

PKA and PKC Regulate Budding of Post-TGN Vesicles via Distinct Mechanisms. Three different types of experimental data indicate that PKA and PKC stimulate formation of vesicles from the TGN via distinct pathways. First, budding of βAPP-containing vesicles was greatly reduced by GTPγS, a nonhydrolyzable analogue of GTP, in the presence of PKA plus OA (Fig. 6, lane 6 vs. 7) but not in the presence of PDBu plus PKC (Fig. 6, lane 8 vs. 9). Second, OA greatly enhanced budding stimulated by PKA in the presence of cytosol (Fig. 6, lane 4 vs. 6) but not that stimulated by PKC (data not shown).

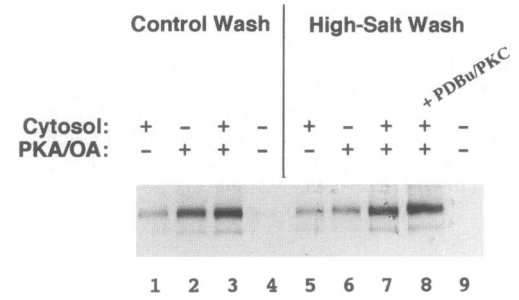

FIG. 5. PKA stimulates vesicle budding from washed TGN-rich membranes. TGN-rich fractions derived from [^{35}S]sulfate-labeled cells were washed with low (100 mM potassium acetate; control) or high (400 mM potassium acetate) salt and analyzed for vesicle budding in the absence or presence of cytosol (1 mg protein/ml) or 25 μM purified PKA catalytic subunit plus 1 μM OA. Lane 8 represents budding in the presence of PKA, OA, cytosol, 1 μM PDBu, and 25 μM PKC.

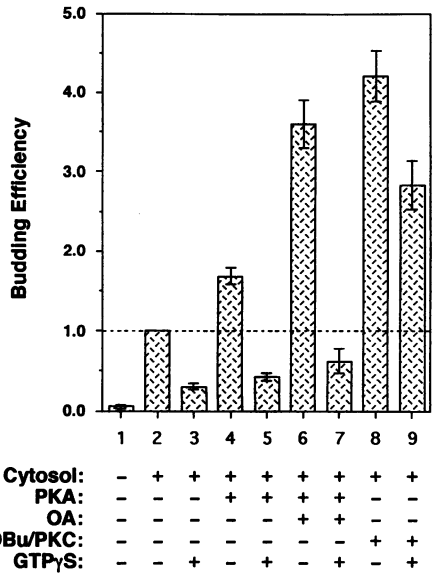

FIG. 6. Effect of GTPγS on PKA- and PKC-mediated budding. The standard budding reaction was carried out under the indicated conditions in the presence or absence of 30 μM GTPγS. Data represent mean ± SEM for three experiments. Lane 2 vs. lane 3, $P < 0.005$; lane 4 vs. lane 5, $P < 0.001$; lane 6 vs. lane 7, $P < 0.005$; lane 8 vs. lane 9, $P < 0.02$.

Third, the effect of optimal concentrations of PKA and PKC were nearly additive in either the presence or absence of cytosol (data not shown).

Implications for Therapeutic Modulation of Amyloidogenesis. Evidence from genetic studies of inherited forms of AD supports the theory that deposition of cerebral Aβ is an early and necessary feature of the disease. Decreasing Aβ production, slowing its aggregation and deposition, or reducing its cytotoxicity are therefore among the most well-grounded therapeutic strategies for preventing this devastating disease and are relevant to all forms of AD. A recent study (18) has directly demonstrated that PKC is involved in regulation of secretory vesicle formation and provides a mechanism by which PKC reduces the formation of the Aβ peptide characteristic of AD. Unfortunately, from a therapeutic standpoint, PKC activation can stimulate βAPP transcription (32), indirectly increasing Aβ formation. The current data suggest that therapeutic agents acting via PKA might circumvent this problem.

We thank Andrew J. Czernik, Atsuko Horiuchi, and Angus C. Nairn for generously supplying purified PKC and PKA. This work was supported by a grant from the C. V. Starr Foundation and Grants AG09464, AG10491, and AG11508 from the U.S. Public Health Service. S.G. is the recipient of a Cornell Scholar Award in the Biomedical Sciences.

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