Amyloid precursor protein (APP) is cleaved predominantly within the β amyloid peptide (BAP) domain to release a non-amyloidogenic amino-terminal PN2 fragment. Treatment of cells with phorbol dibutyrate, an agent which activates protein kinase C, has been shown to increase the release of an amino-terminal fragment. A panel of mutant APP reporter constructs was expressed in which each of the potential phosphorylation sites located within the cytoplasmic domain of APP was replaced with alanine residues. Phorbol response patterns were unchanged for each of these mutants, suggesting that induced cleavage occurs independently of APP substrate phosphorylation. We find that phorbol (a) increases the release of a PN2 fragment that is consistent with the normal secretase activity, (b) decreases the release of a shorter amino-terminal APP fragment that is cleaved near the amino terminus of BAP, and (c) decreases the release of BAP which was identified based on electrophoretic mobility, epitope mapping, and radiolabeling. These data demonstrate that pharmacologic treatment can reduce the formation of BAP and suggests that protein kinase C activators could be developed as therapeutic agents to block BAP formation.

Abnormal extracellular accumulation of amyloid and senile plaques observed in the brain of Alzheimer’s disease patients consists principally of β amyloid peptide (BAP) which is thought to be central to the pathogenesis and memory loss. The 39-42-amino acid (aa) BAP is derived by proteolytic cleavage from a larger amyloid precursor protein (APP), a transmembrane receptor-like glycoprotein which is expressed as three major isoforms containing either 695, 751, or 770 aa (1-9).

The major proteolytic cleavage of APP occurs within the juxtamembranous ectodomain by “secretase” leading to the secretion of an amino-terminal (NH2-terminal) APP fragment (PN2) into conditioned medium (CM). This cleavage takes place within the BAP sequence (between BAP aa residues 16 and 17) and precludes the proteolytic generation of BAP from APP (10-12) which is thought to involve an alternative pathway (13). Release of a shorter NH2-terminal APP fragment following cleavage at the amino terminus of BAP (13), a longer NH2-terminal fragment following cleavage at a distal site carboxy-terminal (COOH-terminal) to the secretase site (14), as well as BAP (15, 16), into CM have all been observed. An endosomal-lysosomal pathway has been suggested to generate potentially amyloidogenic fragments (17-19). Enhancement of the release of BAP into CM is observed by expressing constructs containing the Swedish KM-to-NL mutation, which flanks the NH2 terminus of BAP (20, 21), or 99 aa (C99) derived from COOH-terminal APP sequences, which includes BAP and cytoplasmic APP domains (16).

Activation of protein kinase C (PKC) is known to regulate the secretion (via proteolytic cleavage) or internalization of a number of membrane proteins (22-25). Phosphorylation could be involved in regulation of APP processing and the generation of BAP and amyloidogenic fragments, since the APP holoprotein is phosphorylated (26-30). Treatment of cells with phorbol dibutyrate (PDBu), an agent which activates PKC, increases the release of NH2-terminal APP fragment(s), increases the generation of cell-associated COOH-terminal APP fragments and decreases the amount of mature full-length APP forms, suggesting that substrate APP phosphorylation is involved (26-31).

To better characterize the mechanisms of APP proteolysis, we have developed an APP reporter (APP-REP) as a model system for the expression and cleavage of APP molecules (Ref. 32; Fig. 1A). APP-REP is distinguished from endogenously expressed APP by the deletion of 276 central aa of APP and insertion of the Substance P (SP) reporter epitope in the NH2-terminal ectodomain to enable the immunological detection of PN2 fragments released into CM following proteolytic cleavage of substrate. APP-REP contains 113 aa derived from the COOH-terminal portion of APP and includes intact BAP and flanking sequences, the transmembrane spanning region and the cytoplasmic COOH terminus of APP. In this paper we use APP-REP to determine (a) the nature of the NH2-terminal fragment(s) released from cells after treatment by PDBu, (b) whether increased PN2 release is correlated with a reduction in BAP release, and (c) whether modulation of PN2 release operates by APP substrate phosphorylation. We find that PDBu treatment specifically increases the release of PN2 with a corresponding decrease in both the release of a shorter PN2-like NH2-terminal fragment and BAP. These events occur independently of sub-

The Release of Alzheimer’s Disease β Amyloid Peptide Is Reduced by Phorbol Treatment*

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†† The abbreviations used are: BAP, β amyloid peptide; PN2, NH2-terminal amyloid precursor protein fragment; CM, conditioned medium; aa, amino acid(s); APP, amyloid precursor protein; PRC, protein kinase C; PDBu, phorbol dibutyrate; APP-REP, APP reporter; SP, substance P; Tricine, N,N2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine.

"Secretion" is used only to describe the proteolytic process which results in the release of an NH2-terminal APP (or APP-REP) fragment into the extracellular environment and is not intended to imply or infer any other mechanism.
strate phosphorylation within the cytoplasmic domain of APP. Mutations introduced in the NPYX motif (34) of the cytoplasmic domain of APP increases the release of PNL2 without reducing the formation of BAP, suggesting that internalization is not required for the formation of BAP and that separate pathways may account for secretase and amyloidogenic activities. These findings suggest that the development of therapeutic strategies to activate PKC in cells generating BAP can reduce the formation of BAP.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Transformation, and Plasmid Construction— Transformation, maintenance, and selection of transformants were as described (32). The APP-REP plasmid (pCLL621) represents sequences of APP-751 cDNA with 11 aa of SP reporter epitope sequences replacing a large internal deletion (276 aa) of APP ectodomain (Ref. 32; Fig. 1A).

The Km-to-NL double mutation (i.e. SEVkmDAAEF-to-SEVNLDLE; APP-REP-NL), which affects the two residues immediately NH2-terminal to BAP, and alanine substitutions were introduced into pCCL621 (Figs. 1, A and B) by site-directed mutagenesis using single-stranded plasmid DNA as template as described (33) and resulting mutations confirmed by DNA sequence analysis. C109 was constructed in pCCL621 by substituting an NH2-terminal APP-REP fragment, which corre- sponds to the NH2-terminal APP fragment (XV2-225) fused to the synthetic HindIII-EcoRI fragment. The fragment encodes APP (MLPGLALLLLAAWTARALE) fused to MDAEF (sequences near the NH2-terminal region of BAP) and provides the native signal peptide and cleavage site for the CM and cell lysates. Following cleavage of the signal peptide, a 102-aa protein encoding LE and 100 aa derived from the COOH-terminus of APP is expressed.

Tissue Culture Lines and Transfection—All cells including SV40transformed monkey kidney COS-1 cells (CRL 1650) for transfection, and human embryonic kidney 293 (CRL 1573) and glioblastoma/astrocytoma U-87 MG (HTB 14) cells for stable expression systems, were obtained from American Type Culture Collection and maintained according to their recommendation. Transfection and selection conditions were as described (32).

Radiolabeling of Cells and Preparation of Cell Lysates and Conditioned Medium—APP-REP proteins expressed in exponentially growing monolayers of adherent cells were radiolabeled for the times indicated by the metabolic incorporation of 0.15-0.5 mCi of [3H]methionine (800 Ci/mmol; Amersham Corp.) as described (32). A MeSO solution with or without phorbol dibutyrate (PDBu; Sigma) was added to labeling or chase medium as indicated (final concentrations: 0.05% MeSO with or without 1 μM PDBu). For labeling of cells in suspension, cell monolayers were washed twice with 4 ml of labeling medium (methionine-free medium supplemented with 2% dialyzed fetal bovine serum and 25 mM HEPES, pH 7.4) and incubated for 30 min at 37 °C to deplete methionine. Cells were suspended by gentle trituration, pelleted, and resuspended in 2 ml of labeling medium and incubated at 37 °C with 0.15-0.5 mCi of [3H]methionine for the times indicated. For pulse-chase experiments, an excess of ice-cold chase medium (labeling medium supplemented with 1 μM unlabeled methionine) was then added and the cells washed twice by centrifugation at 4 °C. Labeled cells were then resus- pended in 4°C in fresh chase medium supplemented with a MeSO solution with/without PDBu as before and incubated at 37°C for the times indicated. Supernatants were collected and CM and cell lysates prepared (4 x 106 cells/10-cm culture dish/5 ml of CM or lysate) as described (32).

For radiosequence analysis, two 10-cm culture dishes of 293 cells stably overexpressing the indicated constructs were double-labeled for 6 h by the incorporation of 0.5 μCi of [3H]methionine and 0.5 μCi of [3H]phenylalanine (Amersham Corp.) as described (32). A MeSO solution with or without phorbol dibutyrate (PDBu; Sigma) was added to labeling or chase medium as indicated (final concentrations: 0.05% MeSO with or without 1 μM PDBu). For labeling of cells in suspension, cell monolayers were washed twice with 4 ml of labeling medium (methionine-free medium supplemented with 2% dialyzed fetal bovine serum and 25 mM HEPES, pH 7.4) and incubated for 30 min at 37 °C to deplete methionine. Cells were suspended by gentle trituration, pelleted, and resuspended in 2 ml of labeling medium and incubated at 37 °C with 0.15-0.5 mCi of [3H]methionine (800 Ci/mmol; Amersham Corp.) as described (32). A MeSO solution with or without phorbol dibutyrate (PDBu; Sigma) was added to labeling or chase medium as indicated (final concentrations: 0.05% MeSO with or without 1 μM PDBu). For labeling of cells in suspension, cell monolayers were washed twice with 4 ml of labeling medium (methionine-free medium supplemented with 2% dialyzed fetal bovine serum and 25 mM HEPES, pH 7.4) and incubated for 30 min at 37 °C to deplete methionine. Cells were suspended by gentle trituration, pelleted, and resuspended in 2 ml of labeling medium and incubated at 37 °C with 0.15-0.5 mCi of [3H]methionine (800 Ci/mmol; Amersham Corp.) as described (32). A MeSO solution with or without phorbol dibutyrate (PDBu; Sigma) was added to labeling or chase medium as indicated (final concentrations: 0.05% MeSO with or without 1 μM PDBu).

For radiosequence analysis, two 10-cm culture dishes of 293 cells stably overexpressing the indicated constructs were double-labeled for 6 h by the incorporation of 0.5 μCi of [3H]methionine and 0.5 μCi of [3H]phenylalanine (Amersham Corp.) as described (32). A MeSO solution with or without phorbol dibutyrate (PDBu; Sigma) was added to labeling or chase medium as indicated (final concentrations: 0.05% MeSO with or without 1 μM PDBu). For labeling of cells in suspension, cell monolayers were washed twice with 4 ml of labeling medium (methionine-free medium supplemented with 2% dialyzed fetal bovine serum and 25 mM HEPES, pH 7.4) and incubated for 30 min at 37 °C to deplete methionine. Cells were suspended by gentle trituration, pelleted, and resuspended in 2 ml of labeling medium and incubated at 37 °C with 0.15-0.5 mCi of [3H]methionine for the times indicated. For pulse-chase experiments, an excess of ice-cold chase medium (labeling medium supplemented with 1 μM unlabeled methionine) was then added and the cells washed twice by centrifugation at 4 °C. Labeled cells were then resus- pended in 4°C in fresh chase medium supplemented with a MeSO solution with/without PDBu as before and incubated at 37°C for the times indicated. Supernatants were collected and CM and cell lysates prepared (4 x 106 cells/10-cm culture dish/5 ml of CM or lysate) as described (32).

For radiosequence analysis, two 10-cm culture dishes of 293 cells stably overexpressing the indicated constructs were double-labeled for 6 h by the incorporation of 0.5 μCi of [3H]methionine and 0.5 μCi of [3H]phenylalanine (Amersham Corp.). Immuno-precipitated with antibody 6E10, and immune complexes fractionated by SDS-polyacryl- amide gel electrophoresis (4-20% Tris-Tricine gels; Daichi, Tokyo, Japan) as usual (32). Proteins were then transferred to membrane, visu- alized by autoradiography, and quantitated by laser densitometry. The autoradiograms were then analyzed for radiosequencing. Finally, the antibody binding was quantitated by autoradiography. The antibody binding was quantitated by laser densitometry. The autoradiograms were then analyzed for radiosequencing.

Antiserum and Immunoprecipitation Analysis—Rabbit polyclonal an- tiserum to the SP reporter epitope, immunoprecipitation of radiolabeled lysate or CM, was as described (32). Anti-mouse IgG-agarose (Sigma) was used for the precipitation of monoclonal antibodies 6E10 (anti-BAP1, Ref. 35) and 4G8 (anti-BAP1, Ref. 36) which were raised against synthetic BAP1 and obtained from Drs. K. S. Kim and H. M. Wadiwase (New York State Institute for Basic Research in Developmental Disabilities, Staten Island, NY). Fractionation of immune com-

plexes, autoradiography, and quantitation by scanning laser densitometry or phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA) was as described (32).

RESULTS

Treatment of Cells with PDBu Increases the Release of an NH2-terminal APP Fragment—To more fully characterize the phorbol response of increased APP proteolysis, we used our APP-REP system as a tissue culture model for the expression and cleavage of APP molecules (Ref. 32; Fig. 1A). Human HTB14 (Fig. 2A) and 293 (Fig. 2B) cells stably expressing APP-REP were treated with PDBu and tested for the release of NH2-terminal APP fragments into CM by immunoprecipitation analysis using antibody to SP. In both transfected cell lines, we observe a 3-4-fold increase in the amount of APP-REP-derived ~67 kDa "PN2" fragment in the CM of PDBu-treated cells (Fig. 2A and B, compare lanes 5 with lanes 6). Analysis of corre- sponding cell-associated APP-REP in lysates indicates that PDBu treatment decreases the amount of full-length APP-REP forms (Fig. 2A and B, compare lanes 3 with lanes 2). A similar robust PDBu response is observed with the transient expres- sion of APP-REP in COS-1 cells (data not shown). In pulse-chase experiments (e.g. see Ref. 32, Fig. 4) we find that the effect of PDBu on the progression of post-translational modifi- cations, cleavage of cell-associated full-length APP-REP, and NH2-terminal APP-REP fragment release are nearly identical (data not shown) to those described for APP counterparts (26-31). The presence of 1 μM staurosporine, an inhibitor of PKC, also eliminates the PDBu response with APP-REP (data not shown). In summary, activation of PKC increases the fraction of

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Fig. 2. Phorbol-induced release of PN2 fragment derived from APP-REP. Immunoprecipitation analysis of cell lysate (0.5 ml; lanes 1–3) and CM (0.5 ml; lanes 4–6) from stable expression of APP-REP in (a) HTB14 (human glioblastoma/astrocytoma) and (b) 293 (human embryonic kidney) cells using antisera to SP as described (32). APP-REP proteins expressed in exponentially growing monolayers of adherent cells were radiolabeled by the metabolic incorporation of 0.15 mCi of [35S]methionine in a 15-min pulse, then chased for 0 (lanes 1 and 4) or 2 h (lanes 2, 3, 5, and 6) in the presence (lanes 2 and 6) or absence (lanes 1, 3, 4, and 5) of 1 μM PDBu and unlabeled methionine. Relative amounts of NH2-terminal fragment(s) were determined by scanning laser densitometry. For this and subsequent autoradiograms, molecular mass markers (lane M) are indicated (kilodaltons). Expression of APP-REP initially results in the appearance of two full-length cell-associated forms. An "immature" ~63-kDa form precedes the conversion to a larger ~76-kDa "mature" (i.e. post-translationally modified) form. Subsequent cleavage of APP-REP by secretase releases a shorter ~67-kDa PN2-like NH2-terminal fragment into CM (32).

full-length substrate APP-REP molecules which are rapidly cleaved to release NH2-terminal fragment(s) into CM.

Characterization of the NH2-terminal APP Fragment(s) Released by Treatment with PDBu—We analyzed control CM obtained from the transient expression of APP-REP in COS-1 cells by immunoprecipitation with antibodies to the NH2-terminal portion of BAP and SP reporter (Fig. 1A) to characterize the type of NH2-terminal APP fragment(s) released by treatment with PDBu (Fig. 3). Ordinarily only an ~67-kDa band is visualized (Fig. 3A, lanes 2 and 3), but closer examination of lightly exposed autoradiograms reveals the presence of a doublet band migrating at ~65–67 kDa. Expression of an APP-REP derivative, which is cleaved poorly by secretase (substitution of BAP residues 7–9, with 11 aa derived from another reporter epitope),2 reveals a resolved doublet and a shift in abundance toward the faster migrating component (i.e. ~65 kDa; Fig. 3A, lanes 4 and 5). We then tested the APP-REP fragments released into CM for the presence of the NH2-terminal portion of BAP (i.e. BAP aa residues 1–16) by differential immunoprecipitation with 6E10 (anti-BAP1–16) antibody (Fig. 3B). Immunoprecipitation of CM from untreated control cells with 6E10 yields predominantly the upper component of the doublet (lane 4) as compared with precipitation with SP (lane 3). Immuno depletion of CM with 6E10 (lane 4) and subsequent immunoprecipitation of the remaining CM with SP (lane 5) clearly reveals the lower faster migrating ~65-kDa band. These data are consistent with the report that secretory processing of APP results in the release of classical PN2 containing BAP1–16 as well as a shorter derivative whose complementary cell-associated fragment contains an intact BAP sequence (13). In contrast, when cells are treated with PDBu and the CM is then immunoprecipitated with SP (lane 6) or 6E10 (lane 7), nearly equal amounts are precipitated. Furthermore, if CM immunodepleted with 6E10 (lane 7) is subsequently immunoprecipitated with SP (lane 8), we fail to detect the faster migrating ~65-kDa band. This indicates that PDBu preferentially enhances the release of full-length PN2.

PDBu-enhanced Release of PN2 Is Correlated with Reduced Formation of BAP—To detect and validate the identity of BAP, we analyzed a larger volume of CM either from 293 cells stably expressing APP-695 and APP-REP (Fig. 4) or COS-1 cells transiently expressing the same constructs (data not shown). Immunoprecipitation of CM with 6E10 antibody (Fig. 4A) reveals the presence of ~4.5 (lanes 5 and 7) and ~3.5 (lanes 1–10)-kDa fragments. The ~4.5-kDa fragment is found only in the CM of cells transfected with APP-REP and is uncharacterized. The ~3.5-kDa fragment is detected in CM of all cells but is more abundant in CM of cells transfected with APP-695 (lanes 1 and 2), APP-REP (lanes 5 and 6), or the mutant APP-REP derivative Y743A (lanes 7 and 8) described below. In the presence of competing cold synthetic BAP1–40, the failure to precipitate either fragment with 6E10 antibody indicates both contain an epitope of BAP (data not shown). The ~3.5-kDa fragment (and a shorter ~3-kDa fragment; Ref. 11) is precipitated with 4G8 antibody (data not shown). Therefore, epitope mapping with antibodies having known specificity for BAP sequences and the detection of an ~3.5-kDa fragment in CM from cells overproducing APP-REP and APP-695, an observation similar to that reported by others (16, 37, 38), provides supporting evidence that our ~3.5-kDa peptide is BAP. Radiosequence analysis of ~3.5-kDa fragments derived from a panel of 293 cells stably overexpressing APP-695, APP-REP, APP-REP-NL, and Y743A confirms the identification of the ~3.5-kDa band as BAP (Fig. 4C). The detection of [3H] in the fourth cycle of each fragment analyzed is consistent with the presence of a phenylalanine residue in the fourth position of BAP. Analysis of the corresponding fragment derived from cells overexpressing C1102 indicates the presence of methionine in the third (based on the detection of [35S] and phenylalanine in the seventh aa position exactly as predicted for a construct which, following cleavage of the signal peptide, contains the NH2-terminal extra 3 aa with

2 J. S. Jacobsen and M. A. Spruyt, unpublished data.

Fig. 3. Immunoprecipitation analysis of heterogeneous NH2-terminal APP-REP fragments released into CM from COS-1 cells transiently expressing APP-REP. A, CM (0.5 ml) from cells expressing APP-REP (lane 2), a derivative containing an aa substitution Y743A (lane 3; see Figs. 1B, 4, and 5), a substrate mutant defective in cleavage by secretase (lanes 4 and 5), or vector only control (lane 1) was immunoprecipitated with SP. An autoradiogram representing a short exposure is given to demonstrate the appearance of a doublet band (lanes 2 and 3) which, upon longer exposure, becomes unresolved and is ordinarily observed as a single band (e.g. Fig. 2, A and B, lanes 5 and 6). B, CM from PDBu-treated (lanes 2 and 6–8) or untreated control (lanes 1 and 3–5) cells. APP-REP was pulsed with 0.5 mCi of [35S]methionine for 6 h and CM (0.5 ml) immunoprecipitated with SP only (lanes 3 and 6), 6E10 only (lanes 4 and 7), 6E10 following immunodepletion of CM with SP (lanes 1 and 2, from supernatants of CM following precipitation used in lanes 3 and 6, respectively), or SP following immunodepletion of CM with 6E10 (lanes 5 and 8, from supernatants of CM following precipitation used in lanes 4 and 7, respectively). The band in lane 8 is residual full-length PN2 (~67-kDa fragment) which was not completely immunodepleted from CM with 6E10. A relevant portion of the autoradiograms is shown.
The sequence of leucine (L), glutamic acid (E), and methionine (M) preceeding BAP (Fig. 4D). These data are consistent with the identification of a predominant component of the ~3.5-kDa fragment as BAP.

To determine the effect of PDBu upon formation of BAP, 293 cells were treated with PDBu and CM compared with untreated controls for the presence of immunoprecipitable BAP (Fig. 4A, compare lanes 2 and 1, 5 and 6, and 7 and 8). PDBu reduces the amount of ~3.5-kDa fragment by 60–70% (Fig. 4B) and ~4.5-kDa fragment by 80–90% (data not shown). These data demonstrate that 293 cells normally release BAP into CM and treatment with PDBu causes a significant reduction in release of immunoprecipitable BAP (~3.5-kDa fragment). We observe similar patterns of BAP expression with COS-1 cells (data not shown).

Construction and Expression of APP-REP-based "Phosphorylation-minus" APP Mutants—If phosphorylation of APP is the event which alters processing, mutations introduced at critical sites to prevent phosphorylation should block the observed PDBu response. To construct such phosphorylation-minus derivatives, we substituted each of the 8 aa that are potential phosphorylation substrates located within the cytoplasmic domain of APP-REP with alanine (Fig. 1B). The panel of mutant derivatives were then stably expressed in HTB14 cells. With the exception of Y743A (see below), each mutant releases basal levels of PN2 similar to that of wild type APP-REP, and all typically display a 3–4-fold increase in release of PN2 in response to PDBu (Fig. 5). Quantitation of cell-associated full-length forms indicates similar levels of expression for each mutant construct (data not shown). We observe an identical pattern of PDBu response with wild type APP-REP and the mutant derivatives expressed stably in 293 or transiently in COS-1 cells (data not shown). The fact that phosphorylation-minus mutations continue to be responsive to PDBu indicates that phosphorylation of the APP cytoplasmic domain by PKC (or tyrosine kinase) is not required for PDBu-stimulated release of PN2.

Expression levels of cell-associated full-length Y743A (Fig. 1B) are similar to wild type APP-REP (data not shown). However, the release of Y743A-derived PN2 is ~3–4-fold more than untreated wild type APP-REP controls, whereas addition of PDBu results in only a minimally enhanced release of PN2.

Fig. 4. Production and release of BAP by 293 cells into CM and effect of PDBu treatment on BAP formation. A, immunoprecipitation analysis of CM from PDBu (lanes 1, 3, 6, and 8) or control treated (lanes 2, 4, 5, 7, and 10) 293 cells stably expressing APP-695 (lanes 1 and 2), wild type APP-REP (lanes 5 and 6), a mutant APP-REP derivative containing the Y743A substitution (lanes 7 and 8), or vector only control (lanes 3, 4, 9, and 10). Cells were pulsed with [35S]methionine for 6 h as in Fig. 3B and CM (10 ml) immunoprecipitated with 6E10 antibody. Our BAP (~3.5 kDa) and ~4.5-kDa fragments now migrate more slowly (i.e. at ~4.0 and 5.0 kDa, respectively) relative to markers by SDS-polyacrylamide gel electrophoresis on newer lots of Tris-Tricine 4–20% gels from Daiichi (data not shown). B, effect of PDBu on BAP. The amount of BAP release into CM obtained from 293 cells expressing APP-695, APP-REP, Y743A, or vector constructs (three or four separate experiments) was measured by phosphorimage analysis and is expressed in arbitrary units (mean ± S.E.) relative to that expressed by untreated control (no PDBu treatment). Control (open bar) and 1 μM PDBu-treated (filled bar) samples are indicated. Analysis of variance was used to determine the significance of difference observed between PDBu and untreated control (*, p < 0.0001; **, p < 0.0005). C and D, radiosequence analysis of ~3.5-kDa fragments double-labeled by metabolic incorporation of [3H]phenylalanine and [35S]methionine were immunoprecipitated with antibody 6E10 from CM of 293 cells stably overexpressing APP-695 and APP-REP constructs. The indicated isotope is plotted as counts/min without background adjustment. C, the predominant amino terminus of the ~3.5-kDa fragments derived from APP-REP-NL (●), APP-695 (○), APP-REP (×), and Y743A (□), and their identity as BAP is deduced based on known sequence of BAP; the detection of [3H]phenylalanine, F at the forth cycle and the recognition of BAP, E FRHDS GYE 100, OLEM, and BAP, DFRHDS GYE 100, OLEM, and E FRHDS GYE 100, OLEM, are given at the top. D, the predominant amino terminus of the ~3.5-kDa fragment derived from C130G is deduced based on epitope mapping as in C and the detection of [35S]methionine, M and [3H]phenylalanine, F at the third and seventh cycles, respectively. Sequence representing the NHE-terminal portion of the C130G construct, beginning with the sequence leucine (L), glutamic acid (E), and methionine (M) fused to the NH2 terminus of C130G, is given at the top.
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Fig. 5. Phorbol response in HTB14 cells stably expressing APP-REP and related phosphorylation-minus derivatives. Immunoprecipitation analysis of APP-REP and a panel of phosphorylation-minus derivatives (Fig. 1B) stably expressed in HTB14 cells comparing treatment with PDBu and the release of PN2. Preparation of CM and lanes and immunoprecipitation is as described for Fig. 2, except that APP-REP derivatives were pulsed in suspension, aliquoted, and chased in the presence or absence of PDBu. For three to six experiments, the amount of PN2 (determined by scanning laser densitometry) is expressed in arbitrary units (mean ± S.E. of the mean) relative to that expressed by APP-REP control (no PDBu treatment). Control (filled bar) and 1 μM PDBu-treated (open bar) samples are indicated. Analysis of variance was used to determine the significance of difference observed either for PDBu treatment of individual constructs (*, p < 0.0001) or treated or untreated samples of each APP-REP construct to APP-REP control (**, p < 0.0001).

Discussion

Current understanding of the mechanisms of proteolytic processing of APP suggest that two different pathways may account for the release of (a) PN2, following cleavage by secretary (i.e. "secretase pathway"; Refs. 10 and 11), and (b) BAP, following dual cleavage by activities acting at both NH2- and COOH-terminal sequences of BAP (i.e. "alternative pathway"; Ref. 13). The data presented here add to an understanding of these two processes. The activation of PKC by treatment with phorbol esters enhances the release of NH2-terminal APP fragment(s) which was implied (30, 31, 39) and now shown (this work) to represent PN2 and not a shorter potentially amyloidogenic PN2-like derivative (13). Our demonstration that PDBu, while increasing the release of PN2, simultaneously decreases the release of BAP is significant. The obvious interpretation of our data is that the secretase and alternative pathways compete for APP substrate. However, expression of the mutant APP derivative Y743A and other data requires consideration of other models.

We have clearly demonstrated that PDBu, a known activator of PKC, increases the release of PN2 by a mechanism which is independent of cytoplasmic phosphorylation of APP. Therefore, it is likely that phosphorylation of secretase or other proteins regulates the processing of APP and perhaps other membrane spanning receptors. It is interesting to note that a number of membrane-spanning receptors, such as pro-transforming growth factor-α and the CSF-1 receptor, also undergo rapid ectodomain cleavage and secretion independent of substrate phosphorylation following the activation of PKC by treatment with phorbol ester (40–42). If phosphorylation directly activates secretase, labeling with 32P or binding with PKC (43) may provide an avenue for the characterization, isolation, and cloning of secretase. Alternatively, phosphorylation of an unidentified target may regulate APP processing by increasing access to secretase in a manner which competes with NH2-/COOH- terminal BAP cleaving activities for APP substrate.

The substituted tyrosine of Y743A, which influences APP processing, is located within NPXY, a motif that may be a homolog to the cytoplasmic sequence on the low density lipoprotein receptor which mediates internalization of receptors from the cell surface by coated pit formation (34). This is consistent with the recent observations demonstrating that APP is internalized (17) and localized in clathrin-coated vesicles (44). Deletion of the entire cytoplasmic domain of APP (involving 3 tyrosine residues; Ref. 20), or a portion of cytoplasmic APP sequence (YENPTY; Ref. 45), prevents the internalization of APP and increases the release of PN2. Although these studies imply that NPTY functions similarly to the NPXY motif of low density lipoprotein receptor, the expression of Y743A with a single tyrosine substitution better demonstrates the involvement of the second tyrosine in signaling internalization rather than the first (i.e. YENPTY) as has been proposed (46). These studies support the view that cleavage of APP by secretase may occur on the plasma membrane; however, it has not been demonstrated that failure to internalize results in an increase of APP on the plasma membrane and release of PN2 from that substrate. Furthermore, certain studies suggest that cleavage of APP by secretase may occur within an intracellular compartment (46–48). Alternatively, loss of NPTY function could redirect the transport of more APP to the cell surface by default rather than into the endosome-lysosome pathway (49). In this case, more APP substrate entering the secretase pathway can be cleaved either on the plasma membrane or intracellularly. It is likely that the APP cytoplasmic domain participates in multiple roles pertaining to APP trafficking and processing.

Our data suggest that the inverse relationship between release of PN2 and BAP observed with PDBu treatment is complex as exemplified by the comparison of APP-REP with its mutant derivative Y743A and control or PDBu treatment. Although the same PDBu-induced release of PN2 from APP-REP is not observed with Y743A, we speculate that the already robust release of PN2 with untreated Y743A masks the potential for a further PDBu response due to either saturation of secretase or limited availability of additional substrate. However, elevated release of PN2 with untreated Y743A was not associated with a concomitant decrease of BAP release compared with the untreated APP-REP control. We interpret this lack of an inverse correlation as follows. First, our observations suggest that generation of BAP may not require internalization. Second, cleavage of APP by secretase occurs either subsequent to, or independent of, the generation of BAP. A model consistent with our data is graphically depicted in Fig. 6 to demonstrate these points. The activation of PKC (compare PDBu-treated and control treated conditions, B and D with A and C, respectively) results in the shift of more APP substrate into the secretase pathway, thereby increasing the release of PN2, and less APP substrate into the alternative pathway, thereby reducing the release of BAP. Failure of internalization of APP from plasma membrane enhances the release of PN2 from Y743A (C and D), as compared with APP-REP (A and B), independent of PDBu. Cleavage by secretase is placed distal and independent of the point at which PDBu regulates entry of APP into either pathway to accommodate the release of normal BAP levels despite an increase in release of PN2 from Y743A.
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(53), but not by calcium ionophore A23187 (53). By co-expressing APP-REP and rat m1 receptor, we have confirmed that m1 agonists increase release of PN2 (3) and are testing for the inverse relationship down-regulating the formation and release of BAP. Similarly, interleukin-1, a cytokine that is thought to mediate APP expression via PKC (54), activates a receptor-PKC coupled increase in APP release (39). These observations indicate that receptor-mediated PKC activation, or regulation of the targets of phosphorylation, might be exploited for developing therapeutic interventions that prevent the formation or release of BAP.

It has been reported recently, after completion of this work, that protein phosphorylation inhibits the production of BAP. In that report, compounds known to activate phospholipase C, and therefore PKC, were shown to reduce the generation of BAP in a model which is similar to and supportive of our own (55). However, the actual mechanism responsible for the PDBu response and the apparent inverse relationship between formation of PN2 and BAP is as yet unknown.

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Fig. 6. Schematic illustration of APP processing and the inverse relationship between the release of PN2 and BAP. Expression of APP-REP (A and B) or Y743A (C and D) with PDBu (B and D) or control (A and C) treatment. Represented are two pathways accounting for the release either of PN2 following the cleavage of APP by secretase (i.e. secretase pathway) or BAP following dual cleavage by activities acting at both NH2- and COOH-terminal sequences of BAP (i.e. alternative pathway). Protein phosphorylation of a target other than the cytoplasmic domain of APP is proposed to place more APP substrate into the secretase pathway, thereby increasing the release of PN2 and, as a result, less APP substrate gains access to the alternative pathway, thereby reducing the release of BAP (A and D). Blocking the internalization of APP from plasma membrane increases the release of PN2 without affecting the release of BAP as indicated (C and D). See "Discussion" for a variation of this hypothetical model which accounts for the inverse relationship between the release of PN2 and BAP by a single secretory pathway rather than the dual pathway model illustrated here.
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