Amyloid β Peptide Formation in Cell-free Preparations

REGULATION BY PROTEIN KINASE C, CALMODULIN, AND CALCINEURIN*

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Amyloid β peptide (Aβ) is a short peptide that is the major constituent of the amyloid plaques and cerebrovascular amyloid deposits found in Alzheimer’s disease. The lack of availability of a cell-free system in which to study Aβ formation has limited our understanding of the molecular mechanisms involved in its production. We report here the reconstitution of such a cell-free system. The reconstituted Aβ formation was temperature-dependent and required ATP. Preincubation with purified protein kinase C (PKC) induced a pronounced inhibition of Aβ formation, similar to that observed in intact cells upon stimulation of PKC. The calmodulin antagonists W-7 and trifluoperazine inhibited Aβ formation and enhanced the action of PKC in both the cell-free system and intact cells. A role for the calcium/calmodulin-activated protein phosphatase calcineurin in the regulation of Aβ formation was demonstrated using a specific peptide inhibitor of calcineurin in vitro as well as cyclosporin A, a cell-permeant inhibitor of calcineurin, in intact cells. Our results suggest that a single substrate might mediate opposing actions of PKC and calcineurin in the regulation of Aβ formation.

One of the salient features of Alzheimer’s disease (AD) is neuropathology is deposition of the amyloid β peptide (Aβ) in brain parenchyma and cerebral vessels. This 40–42-amino acid peptide is derived from the Alzheimer amyloid protein precursor (APP) (for a review, see Ref. 1). Mutations in APP have been found to cosegregate with affected status in families with early-onset AD (1). These mutations affect the levels (2–4), length (5), or primary sequence (6) of the Aβ formed. In all of these cases, it has been argued that the mutations would lead to increased Aβ deposition in the brain in a manner sufficient to cause AD. Thus, an understanding of the mechanisms by which Aβ is formed and the means by which Aβ production is controlled may identify avenues for the development of therapies for AD (7).

Numerous studies have demonstrated the formation of Aβ by intact cells (8–10) and its regulation by a number of signal transduction pathways (reviewed in Ref. 11). Activation of protein kinase C (PKC) and/or inhibition of protein phosphatase 1 or 2A dramatically inhibit Aβ formation (12–14). Raising intracellular calcium can, according to the experimental conditions, either inhibit or dramatically stimulate Aβ formation (15, 16). The mechanisms by which these various signal transduction cascades are able to regulate Aβ formation are currently unknown. The availability of a cell-free system in which to study Aβ formation would greatly facilitate the understanding of the molecular mechanisms involved.

We made use of a Balch homogenizer to prepare cracked cells (17). Aβ production in these broken cell preparations was found to depend on ATP and temperature. By introducing purified PKC, a peptide inhibitor of the calcium/calmodulin-dependent protein phosphatase calcineurin, or a calmodulin antagonist (W-7 or trifluoperazine), it was demonstrated that PKC and calcineurin regulate Aβ formation. A role for calmodulin and calcineurin in the regulation of Aβ formation was confirmed in intact cells by the use of cell-permeant inhibitors. The results suggest that PKC and calcineurin bidirectionally regulate Aβ formation, possibly via regulation of phosphorylation of a single substrate.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12,13-dibutyrate (PDBu) (from LC Services Corp., prepared as a 1 mM stock solution in dimethyl sulfoxide), W-7 (from Sigma, 10 mM in deionized H2O), trifluoperazine (from Calbiochem, 5 mM in deionized H2O), cyclosporin A (a generous gift from Drs. G. Snyder and F. Gorelick, 3 mM in dimethyl sulfoxide), calyculin A (from LC Laboratories, 100 μM in dimethyl sulfoxide), and calpain inhibitor I (from Calbiochem, 5 mg/ml in ethanol), were diluted to the indicated final concentrations immediately before use. PKC was purified as described (18).

Preparation of Antibodies 3134C and 3129—Synthetic peptide, corresponding to human Aβ1–40, was used as an immunogen to produce precipitating Aβ antibodies in rabbits. One of the antisera (3134C) was affinity-purified by making use of a synthetic peptide corresponding to residues 22–41 of human Aβ. The affinity-purified antiseraum (3134C), which maps to the carboxyl-terminal domain of Aβ (residues 32–40) and does not recognize secreted APP (sAPP), was used for the ELISA assays. The second antiseraum (3129) immunoprecipitated full-length APP and sAPP, as well as Aβ. This antibody, which does not cross-react with amyloid precursor-like proteins 1 or 2 because of the absence of Aβ domain (1), was used for immunoblot analysis of sAPP.

Sandwich ELISA for the Detection of Aβ—In order to study the regulation of Aβ formation in a cell-free system, a sandwich ELISA was developed. This ELISA makes use of both 3134C as a capture antibody and the monoclonal antibody 6E10 (obtained from Drs. K. S. Kim and H. M. Wisniewski, New York), which is specific for the amino-terminal

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1 The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β peptide; APP, amyloid protein precursor; sAPP, secreted APP; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; APPsw, wild-type human APP; APPg, mutated form of APP found in a Swedish kindred (K670N; M671L; numbering as for APP 770 transcript); ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; GTPγS, guanosine 5’-O-(thiotriphosphate); CHO, Chinese hamster ovary; ANOVA, analysis of variance.

2 F. Lindqvist and C. Nordstedt, unpublished data.
part of human Aβ, as a detection antibody (Fig. 1A). 96-well plates (Nunclon, Maxisorp) were coated for 2 h at 37 °C with 10 μg/ml 3134C in 10 mM carbonate buffer, pH 9.5. Unoccupied binding sites were then blocked with PBS (1 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 1% casein, 0.05% Tween 20, and 0.02% NaN₃. Plates were washed three times in PBS containing 0.05% Tween 20, 0.02% NaN₃ (PBSTA). Test samples (100 μl) were incubated overnight at 4 °C. Subsequently, plates were washed three times in PBSTA, incubated for 2 h with 1–3 μg/ml 6E10 antibody in PBSTA, washed again, and incubated for 1 h with alkaline phosphatase-linked anti-mouse IgG (Southern Biotechnology Associates, Inc.). After three washes in PBSTA, Attphos solution (JBL Scientific, Inc.) was added, and the alkaline phosphatase product was analyzed in a fluorometric plate reader (Millipore Corp.). The ELISA accurately detected >50 pg/ml Aβ, using synthetic peptide corresponding to residues 1–40 of human Aβ as a standard (Fig. 1B).

In some experiments, Aβ was analyzed in conditioned medium from 35S-labeled cells, using both immunoprecipitation of Aβ and the sandwich ELISA. We compared the levels of Aβ in medium derived from CHO cells expressing wild-type human APP (APPwt) or a mutated (K670N; M671L; numbering as for APP 770 transcript) form of APP expressing APPsw, relative to APPwt, and the sand-

\section*{Preparation of Cracked Cells—Monolayers of cells expressing APP were washed once in PBS, resuspended in 5 mM EDTA, centrifuged, washed again in PBS, and resuspended in Buffer H (250 mM sucrose, 15 mM HEPES, pH 7.3, 1 mM magnesium acetate, 1 mM EDTA) to a final concentration of 2 × 10⁶ cells/ml. Cells were then passed once through a 0.00404-inch clearance Balch homogenizer (made by H & Y Enterprise) using a tungsten-carbide ball (Industrial Technicon). Under these conditions, 99.7 ± 0.2% (n = 4) of the cells were trypan blue permeant while still pelleting at 800 × g. These cells, referred to as cracked cells, were incubated (105 cells/ml) at the indicated temperatures in the presence of 2.5 mM magnesium acetate, 20 mM KCl, 0.1 mM CaCl₂, 1 mM dithiothreitol. Energy-depleting (3 mM glucose, 100 units/ml 3134C, an affinity-purified polyclonal antibody used as the capture antibody, recognizes residues 32–40 of Aβ. 6E10 (30) is a monoclonal antibody the epitope of which maps to residues 5–11 of Aβ–40.** B, standard curve using synthetic Aβ–40 (from 0 to 250 pg/ml). Data are expressed as means ± S.E. of three to five standard curves carried out in duplicate. Statistical analysis used an ANOVA test (F₆,₇ = 263.1; p < 0.0001) followed by a Fisher's protected least significant difference test (**, p < 0.01 compared with [Aβ] = 0 pg/ml). Inset, the signal obtained for 10–1000 pg/ml of Aβ (note the semilogarithmic scale). C, cells (20–30 × 10⁶ cells/ml) expressing either APPwt (Wt) or APPsw (Sw) were labeled with [35S]methionine for 2 h and then chased with cold medium for 1 h, as described (12). Aβ in the conditioned medium was either immunoprecipitated with 6E10 (left panel) or detected using the sandwich ELISA (right panel). Immunoprecipitated Aβ was quantified with a PhosphorImager (Molecular Dynamics). Data are expressed as means ± S.E. of four separate experiments. Inset, a typical autoradiogram showing immunoprecipitated Aβ, a.a., arbitrary units.

\section*{Preparation of the reconstituted system—Cracked cells (2 × 10⁶ cells/ml) were centrifuged for 5 min at 1,300 × g at 4 °C. After removal of the postnuclear supernatant, the pellet was gently resuspended in ice-cold Buffer H and incubated for 5 min. This process was repeated three times. After the final resuspension, the cell ghosts were incubated for 15 min at 4 °C before constitution with pretreated postnuclear supernatant. The reconstituted system had about 75% of the Aβ forming activity of the cracked cell preparation (not shown).}

\section*{RESULTS}

\subsection*{Aβ Formation in Cell-free Preparations—Aβ formation in cracked cells was dependent on temperature, with little Aβ measured at 4 °C compared with the amounts formed at 37 °C (Fig. 2A). Formation of Aβ was also dependent on the inclusion of ATP and an ATP-regenerating system (Fig. 2A). Formation of Aβ in cracked cells expressing APPsw or APPwt was linear with time for 30 min, reaching a plateau at about 60 min (not shown). Cracked cells expressing APPsw produced significantly more Aβ than cracked cells expressing APPwt (Fig. 2A), consistent with results observed in intact cells (Fig. 1C). Since the levels of Aβ formed in cracked cells expressing APPwt were near the limit of detection of the ELISA, we used cells expressing APPsw in additional broken cell studies. The Aβ formed was not precipitated during centrifugation at 100,000 × g for 1 h (not shown), indicating that we measured a soluble form of the peptide. As expected from results obtained in intact cells (21), the formation of Aβ was strongly inhibited by the addition of 10 μg/ml calpain inhibitor 1 (28 ± 6% of control, n = 5, p < 0.01), demonstrating that Aβ observed in cracked cells is the result of the cleavage of APP occurring during the incubation period. Aβ formation was not affected by substituting a non-hydrolyzable analog of GTP (GTPγS) for GTP (107 ± 11% of control, n = 4), suggesting that no G-protein was involved in the formation of Aβ in this system. The addition of chloroquine, a weak base that increases the pH of lysosomes and impairs the activity of

\begin{figure}[h]
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\caption{Sandwich ELISA for the detection of Aβ. A, epitope-encompassing regions of the two antibodies used in the sandwich ELISA are shown. 3134C, an affinity-purified polyclonal antibody used as the capture antibody, recognizes residues 32–40 of Aβ. 6E10 (30) is a monoclonal antibody the epitope of which maps to residues 5–11 of Aβ–40.** B, standard curve using synthetic Aβ–40 (from 0 to 250 pg/ml). Data are expressed as means ± S.E. of three to five standard curves carried out in duplicate. Statistical analysis used an ANOVA test (F₆,₇ = 263.1; p < 0.0001) followed by a Fisher's protected least significant difference test (**, p < 0.01 compared with [Aβ] = 0 pg/ml). Inset, the signal obtained for 10–1000 pg/ml of Aβ (note the semilogarithmic scale). C, cells (20–30 × 10⁶ cells/ml) expressing either APPwt (Wt) or APPsw (Sw) were labeled with [35S]methionine for 2 h and then chased with cold medium for 1 h, as described (12). Aβ in the conditioned medium was either immunoprecipitated with 6E10 (left panel) or detected using the sandwich ELISA (right panel). Immunoprecipitated Aβ was quantified with a PhosphorImager (Molecular Dynamics). Data are expressed as means ± S.E. of four separate experiments. Inset, a typical autoradiogram showing immunoprecipitated Aβ, a.a., arbitrary units.}
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lysosomal enzymes (see Ref. 22), did not cause a significant change in Aβ production (94 ± 4% of control, n = 3). The alkylating agent N-ethylmaleimide partially inhibited Aβ formation (71 ± 9% of control, n = 5, p < 0.05), suggesting the involvement of a free sulfhydryl group in Aβ production. For this reason, 1 mM dithiothreitol was present in the standard incubation buffer of the cracked cells.

To confirm the identity of the protein detected by the sandwich ELISA, we labeled cells expressing APPsw with [35S]methionine. The cells were then cracked and incubated for 1 h at 4 or 37 °C in the presence of an energy-depleting (-) or energy-generating (+) system. Aβ was immunoprecipitated from the supernatant using either 6E10 or 3134C in the absence or presence of 5 μg/ml unlabeled synthetic Aβ1–40.

Since stimulation of PKC by PDBu inhibits Aβ secretion in intact CHO cells (12), the effect of purified PKC was tested on Aβ formation in the cell-free system. Cell ghosts expressing APPsw were incubated for 30 min at 37 °C with postnuclear supernatant that had been pretreated for 30 min in the presence of 1 μM PDBu and the indicated amounts of purified PKC. Data are expressed as percentage of Aβ formation in the absence of PDBu and PKC and as means ± S.E. of three to nine experiments. In the absence of PDBu, PKC (3 μg/ml) had no effect on Aβ formation (not shown).

Aβ Formation, but Not sAPP Formation, Is Regulated by Calmodulin and Calcineurin in Intact Cells—To assess whether calmodulin and calcineurin also regulate Aβ secretion in intact cells, cells expressing APPsw were incubated in the absence or presence of PDBu, W-7, trifluoperazine, or cyclosporin A (a specific cell-permeant inhibitor of calcineurin) (24). W-7 and trifluoperazine inhibited the secretion of Aβ from intact cells and potentiated the inhibitory effects of PDBu on Aβ formation (Fig. 6). Cyclosporin A had no effect on the basal secretion of Aβ but potentiated the inhibitory effect of PDBu (Fig. 7). On the other hand, calmodulin antagonists (Fig. 6) and cyclosporin A (Fig. 7) had no discernible effect on sAPP secretion in the absence or presence of PDBu. These results indicate that in intact cells PKC regulates both Aβ and sAPP formation, whereas calcineurin regulates only Aβ formation.

DISCUSSION

One limitation in studying processing and secretion of proteins from intact cells is the difficulty of elucidating the molecular processes involved. In order to better understand the mechanism of action of signal transduction pathways that regulate Aβ secretion, we have developed a cell-free system capable of producing Aβ. Since Aβ formation in intact cells requires both cleavage and secretion steps, we made use of a cell-free system that keeps most of the cytoplasmic ultrastructure and the secretory apparatus intact. This procedure, termed “cell cracking,” was originally developed to preserve the machinery necessary for studying the release of secretory proteins and
Differences in the Aβ formation in broken cell preparations. Cell ghosts expressing APPwt were incubated for 30 min at 37°C with postnuclear supernatant that had been preincubated for 30 min in the absence or presence of PDBu (1 μM)/PKC (3 μg/ml), calyculin A (100 nm), or calcineurin inhibitory peptide (50 μM), as indicated. Data are expressed as percentage of Aβ formation in the absence of any addition and as means ± S.E. of three to six experiments. Statistical analysis used an ANOVA test (F7,25 = 5.554; p = 0.0008) followed by a Fisher’s protected least significant difference test (**, p < 0.01 compared with the control in the absence of any addition; *, p < 0.05 compared with the addition of either calcineurin peptide alone or PKC/PDBu alone).

Fig. 5. Effect of calmodulin inhibitors on Aβ formation in broken cell preparations. Cell ghosts expressing APPwt were incubated for 30 min at 37°C with postnuclear supernatant that had been preincubated for 30 min in the absence or presence of PDBu (1 μM)/PKC (3 μg/ml), W-7 (50 μM), or trifluoperazine (TFP, 25 μM), as indicated. Data are expressed as percentage of Aβ formation in the absence of any addition and as means ± S.E. of three experiments. Statistical analysis used an ANOVA test (F5,12 = 10.195; p = 0.0005) followed by a Fisher’s protected least significant difference test (**, p < 0.01 compared with the control conditions in the absence of treatment; ††, p < 0.01 and †, p < 0.02 compared with the control conditions in the presence of PDBu/PKC alone).

Fig. 6. Effect of calmodulin inhibitors on Aβ and sAPP formation in intact cells. Intact cells expressing APPwt were incubated in Opti-MEM (Life Technologies, Inc.) for 60 min at 37°C in the absence or presence of PDBu (1 μM), W-7 (50 μM), or trifluoperazine (TFP, 25 μM), as indicated. A, Aβ formation. Data are expressed as percentage of Aβ formation in the absence of any addition and as means ± S.E. of four experiments carried out in duplicate. Statistical analysis used an ANOVA test (F7,25 = 180.3; p < 0.0001) followed by a Fisher’s protected least significant difference test (**, p < 0.01 compared with the control conditions in the absence of treatment; ††, p < 0.01 compared with the control conditions in the presence of PDBu/PKC alone). Results similar to those shown here were obtained with cells expressing APPsw. B, sAPP formation. sAPP in conditioned medium was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using serum 3129. This experiment is representative of four independent experiments, each carried out in duplicate.

Regulation of Aβ Formation in Cell-free Preparations

Prolactin from GH3 pituitary cells (17). Our results support the conclusion that Aβ is generated by cracked cells, as opposed to residual intact cells for the following reasons: 1) the dependence of Aβ formation on ATP which, when applied extracellularly, has no effect on Aβ formation in intact CHO cells (16); 2) the effect of the cell-impermeant molecules PKC and the calcineurin-inhibitory peptide on Aβ secretion; and 3) the level of Aβ formed (~10% of that seen in intact cells) compared with the percentage of cells that remained intact (0.3 ± 0.2%).

In agreement with results observed in intact cells, there was much more Aβ produced by cracked cells expressing APPsw as compared withcells expressing APPwt. In fact, it was difficult to detect Aβ formation in cracked cells expressing APPwt, even when using a sensitive sandwich ELISA. For this reason, most broken cell experiments were done with cells expressing APPsw. The question arises as to the relevance of results obtained with these cells for the study of APPwt processing. In experiments in which the two types of transfected cells were compared, they behaved similarly (25). In cell-free preparations, Aβ formation from both APPwt and APPsw was temperature- and energy-dependent. Moreover, the involvement of calcineurin and calcineurin in Aβ formation, observed in cracked cells expressing APPwt, was confirmed in intact cells expressing either APPwt or APPsw.

It will be important to establish whether the regulation of APP processing and Aβ secretion established in CHO cells is also observed in cells derived from the brain (e.g., neurons, astrocytes, microglia, and oligodendrocytes). There is a large and conflicting literature concerning the subcellular localization of the machinery responsible for Aβ formation. Hopefully, the development of cell-free preparations, such as the cracked cell preparation in which Aβ secretion is regulated by the same factors that had been found to regulate Aβ secretion in intact cells, will help to resolve this issue. The present results suggest the existence of a phosphoprotein that stimulates Aβ formation. Identification of this protein and determination of its subcellular site of action should help to clarify the locus of Aβ formation.

Stimulation of PKC (12–14, 26) and inhibition of protein phosphatases 1 and 2A (12, 14) strongly stimulate sAPP secretion and inhibit Aβ formation in intact cells. It was hypothesized that inhibition of the conversion of APP to Aβ might be attributable at least in part to depletion of substrate resulting from activation of the sAPP metabolic pathway (12, 26). In the broken cell preparation used in the present investigation, PKC dramatically inhibited Aβ formation while having no detectable effect on sAPP secretion. These results provide evidence that PKC, in addition to a possible effect on substrate level, has an inhibitory effect on the conversion of APP to Aβ. In addition, calcineurin had no effect on sAPP formation but regulated Aβ formation, both in broken cells and in intact cells. Thus, the effects of calcineurin on Aβ formation appear to be independent of substrate depletion resulting from activation of the sAPP
The analysis used an ANOVA test (F means) and immunoblotting, respectively. Data are expressed as involved in the regulation of A
identification of calcineurin as a major protein phosphatase (28), but not calcineurin, regulate the conversion of APP to sAPP, but not Aβ, secretion, thus providing independent evidence that sAPP and Aβ are derived from distinct metabolic pathways (29). The increasing evidence for such distinct pathways increases the likelihood of developing therapeutic agents capable of inhibiting Aβ formation without affecting sAPP secretion.

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