Therapeutic effects of PKC activators in Alzheimer’s disease transgenic mice

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Alzheimer’s disease (AD) characteristically presents with early memory loss. Regression of K⁺ channels, calcium homeostasis, and protein kinase C (PKC) activation are molecular events that have been implicated during associative memory which are also altered or defective in AD. PKC is also involved in the processing of the amyloid precursor protein (APP), a central element in AD pathophysiology. In previous studies, we demonstrated that benzolactam (BL), a novel PKC activator, reversed K⁺ channels defects and enhanced secretion of APPα in AD cells. In this study we present data showing that another PKC activator, bryostatin 1, at subnanomolar concentrations dramatically enhances the secretion of the α-secretase product sAPPα in fibroblasts from AD patients. We also show that BL significantly increased the amount of sAPPα and reduced Aβ40 in the brains of APP[V717I] transgenic mice. In a more recently developed AD double-transgenic mouse, bryostatin was effective in reducing both brain Aβ40 and Aβ42. In addition, bryostatin ameliorated the rate of premature death and improved behavioral outcomes. Collectively, these data corroborate PKC and its activation as a potentially important means of ameliorating AD pathophysiology and perhaps cognitive impairment, thus offering a promising target for drug development. Because bryostatin 1 is devoid of tumor-promoting activity and is undergoing numerous clinical studies for cancer treatment in humans, it might be readily tested in patients as a potential therapeutic agent for Alzheimer’s disease.

Memory loss, the most characteristic clinical manifestation of Alzheimer’s disease (AD) occurs early in the course of the disease when it primarily affects learning of recent information (1, 2). Previously implicated molecular and cellular processes in the storage of associative memory (3, 4) have also been found affected or de-regulated in cells from AD patients (5, 6). A central and potentially critical locus of convergence between memory acquisition and memory loss in AD is PKC (for general reviews on protein kinase C [PKC] activation and functions, see refs. 7 and 8). PKC has a well established role in memory processes in animal models (9–11) and has been found to be defective in AD (12–14), and Aβ can cause reduction of PKC isoenzymes levels (15–17). In addition, PKC regulates the processing of the amyloid precursor protein (APP) (18–22). Moreover, PKC activation restores K⁺ channel function in cells from AD patients (23).

The processing of APP and its metabolic products plays a fundamental role in AD pathophysiology (24). To have an impact on disease progression, therapeutics must target APP processing to reduce formation of soluble toxic metabolites and, later, to prevent plaque formation. An alternative or complementary approach would target the clearance or degradation of pathogenic APP fragments (24). Identification of the APP-processing pathways and characterization of the key enzymes has provided a framework for pharmacological research on the early pathophysiology of the disease.

APP is a large transmembrane protein that can be cleaved in three distinct sites by proteolytic enzymes collectively referred to as “secretases” (for review, see refs. 24–26). The β-secretase (or BACE) cleaves APP at the amino-terminal side of the Aβ sequence (27), resulting in secreted APPβ and a cell-bound, carboxyl-terminal fragment C99 (also termed C100 or CTF). This fragment is the obligate precursor of the amyloid peptides and substrate for γ-secretase to yield the plaque-forming and neurotoxic fragments Aβ40 and Aβ42 (24–26). In addition, the direct product of β-secretase cleavage, C99, has toxic or pathogenic effects in cultured cells and in transgenic mice (28, 29).

The third enzyme, α-secretase, cleaves within the Aβ sequence to generate a large extracellular soluble fragment (sAPPα) and a smaller intracellular fragment C83 (24, 26). These fragments appear to have no pathological significance and sAPPα might even have neuroprotective properties. Because α- and β-secretases compete for the same pool of APP, promoting the former or inhibiting the latter should result in reduced pathogenic fragments (27, 30). Although considerable effort is being devoted to inhibitors of β- and γ-secretases, less attention has been directed toward α-secretase, for which now tentative candidates have been identified, i.e., ADAM 10 and ADAM17/TACE (26, 31–33).

The “α-processing” of APP is directly or indirectly enhanced by activation of PKC as demonstrated originally with phorbol esters and more recently with novel PKC activators. They have been shown to significantly enhance the secretion of sAPPα (17–23, 34) and reduce Aβ (35, 36). More recently, activation of PKC by PMA has also been shown to prevent Aβ toxicity in rat primary hippocampal neurons (37). Phorbol esters are tumor promoters (38) and, therefore, are not viable options for drug development. Novel PKC activators (18, 23, 39) may offer an alternative, but their safety for eventual human use remains to be demonstrated. A compound that activates PKC and lacks tumor-promoting activity is the natural product bryostatin 1 (40, 41). Bryostatin is being actively investigated in humans (phases I and II) as an anticancer agent (42, 43).

Bryostatin, a macrolide lactone, was first isolated from the bryozoan Bugula neritina by Pettit et al. (44) and recently postulated to be produced by a bacterial symbiont of the bryozoan (45). Bryostatin 1 exhibits high affinity for PKC and displaces phorbol esters at the low nano- and picomolar levels (46, 47). Many other biological effects have also been shown at the subnanomolar level (40, 48). The established lack of tumor promotion and the fact that bryostatin is already in clinical use (46, 47) suggests that it might even have neuroprotective properties. Because α- and β-secretases compete for the same pool of APP, promoting the former or inhibiting the latter should result in reduced pathogenic fragments (27, 30). Although considerable effort is being devoted to inhibitors of β- and γ-secretases, less attention has been directed toward α-secretase, for which now tentative candidates have been identified, i.e., ADAM 10 and ADAM17/TACE (26, 31–33).

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We also extended the previous (18, 23) in vitro studies with novel PKC activators to transgenic AD mice. BL caused a significant increase in sAPP\(\alpha\) and reduction of A\(\beta\)40 in the brain of APP[V717I] transgenic mice. In a follow-up study using the APP[V717I]/PS1[A246E] AD transgenic mice, bryostatin significantly reduced the mortality rate of the transgenic mice and it was effective in reducing both A\(\beta\)40 and A\(\beta\)42.

**Materials and Methods**

**Cells and Cell Culture.** Two well characterized cell lines from AD patients, AG60848 (familial case, autopsy confirmed) and AG07377 (sporadic case), and two age-matched controls (AG60711 and AG066241) were obtained from the Correll Cell Repository (Camden, NJ; http://locus.umdnj.edu/ccr). Cells were subcultured and maintained as described (18). In brief, cells were seeded in Petri dishes containing 2 ml of DMEM (Life Sciences) supplemented with 10% FBS and grown to confluence in a 37°C/5% CO\(_2\) incubator for 5–7 days.

**Measurements of sAPP\(\alpha\).** Culture medium was replaced with serum-free medium 2 h before treatment. Bryostatin (Biomol, Plymouth Meeting, PA), BL (Shanghai Institute of Organic Chemistry and Georgetown University Drug Discovery Program, Plymouth Meeting, PA), and vehicle control (DMSO, Aldrich) were added directly to the dish by pipette to achieve the final desired concentration (0.1 and 0.01 mM for bryostatin; 0.1 mM, 0.1 \(\mu\)M, and 1 \(\mu\)M for BL). DMSO was <1% in all cases. Treatment was maintained for 3 h, except for time-course secretion experiments where 5-, 30-, 60-, 120-, and 180-min data points were taken. Pretreatment (30 min before bryostatin) with 100 nM staurosporin (Sigma) was also conducted. Upon completion of the treatment, medium was collected, precipitated with 20% trichloroacetic acid, spun at 14,000 \(\times\) g for 1 h. The pellet was then resuspended in acetone and spun again for 1 h at 14,000 \(\times\) g. After decanting the acetone, the pellet was resuspended in Laemmli electrophoresis sample buffer. The samples were boiled for 5 min, subjected to electrophoresis and later immunoblotting for identification of sAPP. After conventional immunoblotting techniques, precipitated protein extracts from each cell line and condition were loaded to freshly prepared 10% acrylamide Tris\(\cdot\)HCl minigels and separated by SDS/PAGE. Proteins were then transferred electrophoretically to poly(vinylidene difluoride) membranes. The membranes were saturated with 5% nonfat dry milk to block nonspecific binding. Blocked membranes were incubated overnight at 4°C with 200 ng per ml of antibodies for PKC isoforms (Transduction Laboratories, Lexington, KY) followed by exposure to Kodak Biomax MR (Kodak). The band intensities were quantified by densitometric analyses by MULTIANALYST software (Bio-Rad).

**PKC Translocation.** Measurements of cytosolic and membrane-bound levels of isoenzymes was used to assess PKC translocation in response to bryostatin treatment (0.1 nM), using procedures slightly modified from those established by Racchi et al. (50) and Ibarreta et al. (18). After 2 h of serum deprivation, cells were treated for 5, 30, 60, and 120 min. Fibroblasts were then washed twice with ice-cold PBS, scraped in PBS, and collected by low-speed centrifugation. The pellets were resuspended in the following homogenization buffer: 20 mM Tris\(\cdot\)HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 5 mM DTT, 0.32 M sucrose, and protease and phosphatase inhibitor mixture (Sigma). Homogenates were obtained by sonication and centrifuged at \(~12,000\times\) g for 20 min, and the supernatants were used as the cytosolic fractions. The pellets were homogenized in the same buffer containing 1.0% Triton X-100, incubated in ice for 45 min, and centrifuged at \(~12,000\times\) g for 20 min. The supernatant from this batch was used as the membranous fraction. After protein determination, 20 \(\mu\)g of protein was diluted in 2× electrophoresis sample buffer (Novern, San Diego), boiled for 5 min, run on 10% acrylamide gel, and transferred electrophoretically to a poly(vinylidene difluoride) membrane. The membrane was saturated with 5% milk blocker by incubating it at room temperature for 1 h. The primary antibodies for PKC isoforms (Transduction Laboratories, Lexington, KY) were diluted (1:1,000) in blocking solution and incubated with the membrane overnight at 4°C. After incubation with the secondary antibody, horseradish peroxidase conjugated anti-mouse IgG (The Jackson Laboratory), horseradish peroxidase- bind signal was detected by using enhanced chemiluminescence (Pierce) followed by exposure to Kodak Biomax MR (Kodak).

**Transgenic Animals and in Vivo Studies.** Transgenic mice carrying the APP[V717I] mutation were generated and characterized as described (51, 52). Animals (n = 4) were treated with BL (1 mg/kg i.p. daily) from 5 weeks of age (after weaning) for 17 weeks. The control group (n = 4) received vehicle alone (1% Tween 20/25% DMSO/74% PBS). Another experimental group consisted of 5- to 6-month-old animals treated for 7 weeks. Subgroups of these animals were treated with BL, 1 mg/kg daily (n = 5); BL, 10 mg/kg daily (n = 3; due to two deaths); BL, 10 mg/kg weekly (n = 4; one death); LQ12 10 mg/kg daily (n = 5); and LQ12, 10 mg/kg weekly (n = 5). LQ12 is a PKC activator developed by Quiao et al. (53). Five additional animals received vehicle alone for the same period. Standard general health assessment and open field were conducted in all animals before the biochemical assessments. In addition, a semiquantitative ad hoc score was devised to measure abdominal contractions that followed the injections (+, weak, \(~\pm\)min; +++, strong, \(~\pm\)1.2 min; +++, very strong, \(~\pm\)1.2 min). After completion of the treatment, animals were killed according to Katholieke Universiteit Leuven guidelines. Brains were removed and prepared for biochemical analyses of APP species.

A second group of 56 animals initially was progressively reduced because of premature death. Consequently, the size of the various experimental groups analyzed varied depending on the number of surviving animals (details in each section). These transgenic animals carry the same APP[V717I] mutation plus PS1[A246E] (development and characterization described in ref. 52). They were treated with bryostatin 40 \(\mu\)g/kg i.p. injections three times per week from \(~3\) weeks of age (just after weaning) to \(~5\) to 6 months, unless spontaneous death occurred earlier. Controls received vehicle alone (1% Tween 20/25% DMSO/74% PBS). A total of 30 animals (14 controls and 16 treated) survived until the end of the study. The duration of the study was shortened from its original design (11 months) because of elevated mortality, in particular, in the transgenic control group. Therefore, a subset of the surviving cohort of animals received treatment or vehicle alone for a shorter period, ranging from 20 to 24 weeks.

**Biochemical Analysis of APP Processing in Brain of APP and APP/PS1 Transgenic Mice. Immunoblot analysis.** The biochemical analysis of intermediates of APP metabolism has been described by Dewachter et al. (29). In brief, brains were homogenized in 6.5 vol of ice-cold buffer containing 20 mM Tris\(\cdot\)HCl (pH 8.5) and a mixture of proteinase inhibitors (Roche, Darmstadt, Germany). After centrifugation at 13,000 \(\times\) g at 4°C for 1 h, the supernatant was centrifuged again for 2 h at 200,000 \(\times\) g before analysis of soluble amyloid peptides by specified ELISA. The pellets from the first
centrifugation were resuspended in TBS containing 2% Triton X-100, 2% Nonidet P-40, and proteinase inhibitors and centrifuged at 100,000 × g at 4°C for 1 h. This protein fraction was used for analysis of membrane-bound APP. Western blotting of membrane-bound APP was performed on this protein fraction containing membrane-bound proteins, with mAb 8E5. Total secreted APP and α-secretase cleaved, secreted APP-α, were detected by Western blot analysis on the supernatant of the first centrifugation, with mAb 8E5 or 6E10 and mAb JRF14, respectively. Proteins were denatured and reduced in sample buffer containing a final concentration of 2% SDS/1% 2-mercaptoethanol and separated on 8% Tris glycine gels (Novex). After incubation with appropriate secondary antibodies, all Western blots were developed with the enhanced chemiluminescence detection system and photographically recorded. Application of a series of diluted samples allowed quantitation. Densitometric scanning of films and normalization were performed as described (51, 52) by using a flatbed optical density scanner and dedicated software for analysis and measurement (Image Master, Pharmacia, Uppsala).

**ELISA of amyloid peptides.** Protein extracts were applied on reversed-phase columns (C18-Sep-pak cartridges; Waters) and washed with increasing concentrations of acetonitrile (5%, 25%, and 50%) containing 0.1% trifluoroacetic acid. The last fraction contained the amyloid peptides and was dried in vacuo overnight and dissolved for measurements in ELISA. Sandwich ELISA for human Aβ40 and Aβ42 peptides in the single transgenes was performed with the capture antiserum R1/F/aβ40/10 and 21F12, respectively, and they were developed with mAbs JRFαAbtot/14hrdo and 3D6, respectively (54). In the double transgenes, sandwich ELISA for human Aβ40 was similar, but sandwich ELISA for human Aβ42 was performed with an ELISA kit for human Aβ42 (Innogenetics, Ghent, Belgium).

**Analyses of amyloid deposits.** Histological analyses of amyloid deposits included thioflavin S staining and immunohistochemistry with anti-Aβ antibody. Details of the procedures are described elsewhere (29, 51, 52).

**Behavioral analyses of APP [7171] /PS1[A246E].** Animals completing the treatment underwent both Open Field and Morris Water Maze testing according to standard procedures adapted to mice (51, 55).

**Results**

**sAPPα Secretion in Fibroblasts.** Treatment of AD cell line AG06848 with 0.1 nM bryostatin for 3 h dramatically increased secretion of APPα (overall ANOVA, P < 0.0001; Tukey’s posttest comparison to DMSO alone, P < 0.001) (Fig. 1, solid bar). A similar effect was obtained with the AD cell line AG07377 (secretion in normalized units = 32.48 ± 2.5). This result, together with previous demonstrations of similar effects of PKC activation on several lines of familial and sporadic AD cases (18, 23), permitted us to focus the majority of the experimental procedures on a particularly well characterized cell line (AG06848).

The effect of bryostatin 1 was also significantly higher than another PKC activator (BL) used at the same (0.1 nM) or higher (1 μM) concentration, P < 0.001 and P < 0.05 (Tukey’s posttest), respectively. Pretreatment with 100 nM staurosporin, a PKC blocker, abolished the effects of 0.1 nM bryostatin (Fig. 1, rightmost bar). In cell lines from two age-matched controls (poled), bryostatin (0.1 nM) also significantly (compared to DMSO alone, P < 0.01, Tukey’s posttest) enhanced the secretion of sAPPα, but to a significantly lesser extent than in the AD cell line (Fig. 1, hatched bar; P < 0.05, Tukey’s posttest). A time-course experiment (Fig. 1 Fig. 1 Insert) showed a marked increase in sAPPα secretion after 15 min of incubation with 0.1 nM bryostatin. Progressive and proportional increases were observed at 30 and 60 min. Incubation periods of 2 and 3 h did not substantially differ from 60 min of incubation in terms of the amount of APPα secreted. The lower concentration of bryostatin, 0.01 nM, produced a robust enhancement of sAPPα secretion only after 60 min of incubation. The effect of the low concentration (0.01 nM), however, was indistinguishable from the higher (0.1 nM bryostatin) at 2 and 3 h of incubation (Fig. 1 Insert). Bryostatin (0.01 nM) for 3 h was equally effective in the AD cell line AG07377 (not shown).

**PKC Translocation.** The distribution of cytosolic and membrane-bound forms of the PKC-α isoenzyme was determined at various time points after incubation with bryostatin. A relative increase (compared with DMSO alone) in the ratio particulate/soluble (P/S) immunoreactivity was consistent and significantly different from DMSO alone (P = 0.0411; Student’s t test, two-tailed) after 30 min of incubation (Fig. 2). The P/S ratio declined progressively, but it remained higher, albeit not statistically significant, than in cells treated with DMSO alone, after 180 min.

![Fig. 1.](NEUROSCIENCE)\textcopyright{} PNAS 2004 101 no. 30 11143

![Fig. 2.](NEUROSCIENCE)\textcopyright{} PNAS 2004 101 no. 30 11143
of incubation (Fig. 2A). The effect of 0.01 nM bryostatin was much less marked and developed more slowly, with a maximum P/S ratio value at 120 min of incubation. Translocation of other PKC isoenzymes was assessed at 30 min of incubation with 0.1 nM bryostatin by immunoreactivity with specific antibodies for ε-, β-, and δ-isoenzymes. In all cases, the ratio S/P was higher than DMSO alone and comparable with the levels of PKC-α (Fig. 2B).

Transgenic APP[V717I] Mice. Transgenic mice (n = 4) were treated with BL (1 mg/kg) from weaning (age, 3 weeks) for 17 weeks. In their brains, a significant (P = 0.04, Student’s t test) increase in sAPP-α and a concomitant and significant (P = 0.02, Student’s t test) reduction in Aβ40 level were evident, compared with controls (n = 4) treated with vehicle alone (Fig. 3). No differences were noted in the amount of Aβ42, of total membrane-bound APP, or of total secreted APP (sAPPα + sAPPβ) (results not shown). No differences in general appearance or in body weight became evident during this period, despite the fact that the i.p. injections caused some abdominal contractions that were reversible and similar in frequency in both groups.

Another group of mice (22 treated and 5 controls; see Materials and Methods for subgroup details) treated with BL and LO12 beginning at 6 months of age and for a shorter period (7 weeks) showed a similar general trend but the changes in sAPPα and Aβ40 were small and did not reach statistical significance. Similarly, no significant changes in Aβ42, total soluble APP, or in membrane-bound APP were noted in these mice (results not shown).

Abdominal contractions and flaccidity of the hind legs were observed in the older animals upon injections, which were completely reversible within 3–12 h. These contractions appeared somewhat related to the dose, but objective assessment was not possible. The general health and the body weight of all mice, treated or untreated, were normal. In total, three mice died (7.8% of the total) during the course of the experiments from causes that did not appear to relate directly to the treatment.

Transgenic APP[V717I]/PS1[A246E] Mice. Based on the previous results indicating that PKC activation was effective only if treatment started early in life, this entire group (n = 56) of transgenic animals began treatment from weaning (∼3 weeks old). Mice received 40 μg/kg of bryostatin or vehicle alone by i.p. injection three times per week. The duration of the treatment was influenced by the mortality rate. Fourteen controls and 16 treated animals completed an abbreviated protocol lasting an average of 5.5 months (range, 4.7–5.63 months). Twenty-six mice died during the course of the study at various time points, preventing their use for behavioral, biochemical, or histopathological analyses.

Mortality. Premature death, as documented (51, 56), was observed in both treated and control (receiving vehicle alone) animals. However, noticeable differences occurred between treated and control animals. Of all the mice monitored (n = 52), ∼70% of those treated mice reached ∼7 months of age. In contrast, only 48% of the control animals were alive at 7 months of age; in fact, most of these animals died (52%) just before reaching 6 months of age (Fig. 4A). Despite the marked differences, statistical significance for the entire group was not reached (P = 0.16, Fisher’s exact test, two-tailed). Analyses by gender, however, showed robust differences and explained the lack of statistical significance when males and females are analyzed together. Only 1 of 13 control males reached 7 months of age; in fact, nearly 80% were dead by 5 months of age (Fig. 4B). Six of 9 treated males (∼67%) reached 7 months of age (compared with the survival of controls, P = 0.006, Fisher’s exact test, two-tailed). In contrast to these observations in males, both treated and control females showed similar survival rates; 10 of 14 treated and 13 of 16 untreated females reached 7 months of age (P = 0.67, Fisher’s exact test, two-tailed) (Fig. 4C). In addition, females as a group (treated and untreated) showed significantly

Fig. 3. Transgenic mice APP[V717I]. Mice (four treated and four controls) began treatment form just after weaning (3 weeks) with BL (1 mg/kg i.p., daily) for 17 weeks. (A) sAPP-α increased significantly (P = 0.04) in the brains of the treated group compared with vehicle alone. (B) The same animals had a proportionally significant (P = 0.02, Student’s t test) reduction of Aβ40.

Fig. 4. Transgenic mice APP[V717I]/PS1[A246E]: Mortality. (A) Life table analysis of all mice. Both treated (n = 23) and untreated (n = 29) mice showed premature death; however, the rate was slowed by bryostatin. (B) Life table analysis of male mice. The difference was particularly salient in males (n = 22). Nontreated animals exhibited elevated mortality that was significantly reduced by bryostatin (P = 0.006, Fisher’s exact test, two-tailed). (C) Life table analysis of female mice. Females (n = 30) did not show the same level of premature death, and treatment did not change the rate significantly.
higher survival rate than males by 7 months ($P = 0.001$, Fisher’s exact test, two-tailed).

**Amyloid peptides.** No significant differences occurred in total full-length APP between treated and untreated groups ($P = 0.08$, Student’s $t$ test; two-tailed). There was a clear, albeit not statistically significant reduction in the amount of soluble $\beta$-amyloid in the treated group. The treated group exhibited a 37% reduction in $\beta$-amyloid compared with controls (Fig. 5A). Corrected or normalized data by full-length APP shows an even greater reduction of $51\%$. $\beta$-amyloid was also reduced in the treated group. The treated group (Fig. 5B) showed a reduction of 58.6% compared with controls ($P = 0.04$, $t$ test, one-tailed). Normalized data show a greater reduction of 65.3% but did not reach statistical significance. Aggregated $\beta$-amyloid detected by immunohistochemistry or thioflavin S also shows reductions (40%) in the treated group; however, the differences did not reach statistical significance (data not shown). Measurements of percent of amorphous plaque per section showed the same trend (not shown).

**Behavior.** No significant improvements occurred in the spatial maze learning of treated mice compared with controls in the Morris water maze. Both groups appeared impaired and unable to learn the task and spent more time in the quadrant opposite to the target (not shown). The treated group showed a slight but not significantly increased frequency in the target quadrant in a corrected prove test (data not shown). In the open field test, the treated animals ($n = 15$) spent more time in the border area and reduced time in the center compared with vehicle-only controls ($n = 14$; $P < 0.04$, Student’s $t$ test in both cases). These results may be indicative of a more appropriate neophobic reaction or, alternatively, due to increased anxiety in the treated group. Frequency in border and center were, however, not different between groups.

**Discussion**

These results provide evidence for a significant role for PKC in AD pathophysiology. We have confirmed reports by ourselves and several other groups (17–23, 32, 34) that activators of PKC lead to increased processing of APP by the $\alpha$-secretase pathway. The demonstration of a potent $\alpha$-secretase activation effect of bryostatin, an activator already in clinical use (42, 43), suggests that modulation of the $\alpha$-secretase pathway may be a plausible therapeutic strategy. It is also important to point out that the effect of bryostatin on control cell lines was less marked than in the AD cell line. This finding is not only consistent with our results using BL (23), but it also suggests that the effect of bryostatin will be greater in a pathology–sensitive system.

The role of PKC activation mediating the effect of bryostatin on APP metabolism was demonstrated by abrogation of its effect by pretreatment with staurosporin, a well known PKC blocker. We also showed that several isoenzymes are activated, as measured by translocation from cytosol to the membrane-bound fraction. It has already been established that $\alpha$ and $\epsilon$ play a role in APP processing (18, 34). It is also known that the $\delta$-isofrom may be the key for the anticancer properties of bryostatin (41, 57). However, it remains unclear whether its activation and the activation of $\beta$ (translocation) played a role in the enhanced $\alpha$-secretase processing of APP in fibroblasts. Some reports have shown rapid PKC$\alpha$ down-regulation with bryostatin (47). Our data, however, show a variable translocation at 5 min and a more consistent translocation at 30 min. The levels decreased but, in general, remained higher than DMSO alone up to 3 h later.

We have also expanded our previous data on the in vitro enhancement of sAPP$\alpha$ secretion by BL (18) and the present bryostatin results to two separate in vivo studies. Using a transgenic model of AD, we have shown that BL also enhances sAPP$\alpha$ secretion in vivo, with a proportional reduction of $\beta$-amyloid only if the treatment is initiated early in life (immediately after weaning), presumably before the emergence of major APP-processing changes. Therefore, increase of one enzymatic pathway necessarily results in less substrate for an alternative enzyme. In this case, a reduction of amyloid and toxic fragment (A$\beta$40) is achieved by increasing the nonpathogenic $\alpha$-secretase processing of APP. The fact that the total secreted APP ($\alpha$- + $\beta$-products) was not different between treated and untreated animals is consistent with and confirms the previous interpretation. It is also apparent that the increase in sAPP$\alpha$ is not the result of elevated total APP (or increased expression), because membrane-bound APP is similar in both treated and control groups. The lack of effect on BL on the amount of A$\beta$42 is not readily explained. However, A$\beta$42 is a minor fraction of the total A$\beta$ (30, 32, 51, 52), and activation of PKC is not expected to influence $\gamma$-secretase activity. In addition, the assay may have not been sufficiently sensitive to detect small effects.

A follow-up study in the double transgenic animals with accelerated pathology largely confirmed the previous findings and also demonstrated a clear effect on A$\beta$42. Aggregated amyloid (amorphous plaques) was also found reduced in treated animals. Although the latter result and the reduction of A$\beta$40 did not reach statistical significance (most likely because of mortality and resulting reduced sample), the trend is clear, and it cannot be attributed to changes in total APP. Bryostatin also significantly reduced the elevated mortality common to these transgenic animals (51, 56). The latter was an unexpected “beneficial” effect of the bryostatin treatment. The transgenic mice have a significantly higher mortality rate than nontransgenic strains, but otherwise they have the same genetic background. The elevated mortality has been linked to the expression of human APP. The mechanism by which bryostatin exerted its protective effects remains unknown, but it is reasonable to speculate that it relates to an enhanced (and perhaps accelerated) processing of APP by the $\alpha$-secretase pathway. It is also interesting that males had higher rates of premature mortality than females. This finding seems opposite to observations in other transgenic models and human AD showing that females are more affected (58). However, females used here are indeed more affected than males in terms of amyloid burden (S.W., unpublished data). The gender effect on mortality showed in this study, while not readily explained, has been observed previously and should undergo further characterization (S.W., unpublished data).

The most marked “beneficial” effect was observed in animals that had begun treatment early in life and for a longer period. This finding suggests that preventing long-term effects of toxic fragments should be an important goal of therapy. Intervention later in life and later in the course of the disease process (even without clinical manifestations), as suggested by the results obtained in older mice, would have much less impact in preventing damage by toxic fragments. Also, the transgenic models used in this study (29, 51, 52) and first, also have transgenic models and cognitive deficits and, later, amyloid deposition with plaques and angiopathy. In agreement with in vitro studies (for review,
in vitro this sequence of events shows that soluble amyloid species can be deleterious, presumably at intracellular loci in the soluble form, before any significant extracellular deposition occurs. Although longer-term studies would be required for conclusive evidence, it is reasonable to speculate that early treatment with PKC activators will prevent cognitive deficits associated with amyloid peptide load and plaque burden.

PKC has long been implicated in memory mechanisms (9–11), both in invertebrates and mammalian models. Some evidence also exists that secreted APP may by itself improve memory in normal and amnestic mice (59). Therefore, PKC appears to both in invertebrates and mammalian models. Some evidence 11146–11147 suggests that treatment with PKC activators will prevent cognitive deficits seen ref. 6), this sequence of events shows that soluble amyloid. Although the behavior studies did not indicate improvements, a longer-term study would be necessary to completely assess cognitive and other behavioral effects, given the high mortality of the double-mutation transgenic strain.

Modulating PKC is already a clinical reality in oncology (42, 43) and in some psychiatric conditions, where PKC inhibitors are undergoing clinical trials (61). Therefore, modulating APP metabolism by PKC activation, in particular, with isoenzyme specificity, offers a plausible approach for developing AD therapeutics. Bryostatin, given its lack of tumor promotion activity, relatively low toxicity, and current use in humans, may be a suitable candidate for further drug development.

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