Amyloid β -peptide disrupts carbachol-induced muscarinic cholinergic signal transduction in cortical neurons

(acetylcholine/Alzheimer disease/antioxidant/calcium/carbachol)

JEREMIAH F. KELLY*, KATSUTOSHI FURUKAWA†, STEVEN W. BARGER†, MOHAN R. RENGAN*, ROBERT J. MARK†, EMMANUELLE M. BLANC†, GEORGE S. ROTH*, AND MARK P. MATTSON†‡

*Molecular Physiology and Genetics Section, Gerontology Research Center, National Institute on Aging, Baltimore, MD 21224; and †Sanders–Brown Research Center on Aging and Department of Anatomy and Neurobiology, University of Kentucky, Lexington, KY 40536

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ABSTRACT Cholinergic pathways serve important functions in learning and memory processes, and deficits in cholinergic transmission occur in Alzheimer disease (AD). A subset of muscarinic cholinergic receptors are linked to G-proteins that activate phospholipase C, resulting in the liberation of inositol trisphosphate and Ca^{2+} release from intracellular stores. We now report that amyloid β -peptide ($\text{A}\beta$), which forms plaques in the brain in AD, impairs muscarinic receptor activation of G proteins in cultured rat cortical neurons. Exposure of rodent fetal cortical neurons to $\text{A}\beta_{25-35}$ and $\text{A}\beta_{1-40}$ resulted in a concentration and time-dependent attenuation of carbachol-induced GTPase activity without affecting muscarinic receptor ligand binding parameters. Downstream events in the signal transduction cascade were similarly attenuated by $\text{A}\beta$. Carbachol-induced accumulation of inositol phosphates (IP_1 , IP_2 , IP_3 , and IP_4) was decreased and calcium imaging studies revealed that carbachol-induced release of calcium was severely impaired in neurons pretreated with $\text{A}\beta$. Muscarinic cholinergic signal transduction was disrupted with subtoxic levels of exposure to $\text{A}\beta$. The effects of $\text{A}\beta$ on carbachol-induced GTPase activity and calcium release were attenuated by antioxidants, implicating free radicals in the mechanism whereby $\text{A}\beta$ induced uncoupling of muscarinic receptors. These data demonstrate that $\text{A}\beta$ disrupts muscarinic receptor coupling to G proteins that mediate induction of phosphoinositide accumulation and calcium release, findings that implicate $\text{A}\beta$ in the impairment of cholinergic transmission that occurs in AD.

Cholinergic pathways projecting from basal forebrain regions to hippocampus and neocortex are believed to play important roles in learning and memory processes (1). In Alzheimer disease (AD) cholinergic systems are disrupted, and neurons ultimately die in several connected brain regions including hippocampus and basal forebrain (2). However, it is unclear whether deficits in cholinergic signaling occur prior to neuronal loss. Acetylcholine binding to muscarinic cholinergic receptors initiates the heterotrimeric G protein cycle, which commences with the exchange of GTP for GDP on α -subunits and the subsequent dissociation of $\beta\gamma$ subunits. The activated, GTP-bound form of the α -subunit stimulates (or inhibits) its effector, then undergoes inactivation by intrinsic GTPase activity, which converts GTP to GDP by hydrolytic cleavage of the γ phosphate bond. Cholinergic agonist stimulation of M_1 , M_3 , and M_5 receptors activates G proteins of the pertussis toxin-insensitive $\text{G}_{q/11}$ family. $\text{G}_{q/11}$ subunits stimulate phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate, resulting in the liberation of diacylglycerol and inositol triphosphate (IP_3). Diacylglycerol activates protein kinase C, and IP_3 induces the release of Ca^{2+} from endoplasmic reticulum (3).

Studies of postmortem brain tissue obtained from AD patients have shown evidence of impaired coupling of muscarinic receptors to G proteins (4–7). For example, Flynn *et al.* (5) reported a reduction in the proportion of M_1 muscarinic receptors existing in the high-affinity (G-protein coupled) state in AD frontal cortex, compared with age-matched controls. This apparent defect in coupling may, in part, explain the lack of efficacy of cholinergic agents in ameliorating cognitive symptoms of AD. Not all investigators have detected such changes in AD (8), and the explanation for the discrepancies in study findings may be related to differences in brain region chosen or in relative severity/duration of disease (9). Additional data suggest coupling abnormalities in other receptor-G protein-effector systems in AD including a reduction in the high-affinity form of the D_1 dopamine receptor (10), deficits in adrenergic receptor coupling to G_s and adenylate cyclase production (11–14), and alterations in β -adrenergic receptor coupling in cultured fibroblasts from AD patients (15).

The amyloid β -peptide ($\text{A}\beta$) is a 39–43 amino acid peptide, derived from a much larger membrane-associated β -amyloid precursor protein (βAPP) that forms insoluble plaques in the brains of AD victims (16). $\text{A}\beta$ can be neurotoxic (17) by a mechanism that involves generation of free radicals and disruption of cellular Ca^{2+} homeostasis, resulting in elevated rest [Ca^{2+}], and increased sensitivity to excitatory amino acids and membrane depolarization (18–25). When cultured hippocampal neurons are exposed to $\text{A}\beta$, the peptide accumulates on or in the plasma membrane (20), which appears to be a major site at which $\text{A}\beta$ induces oxidation of lipids and proteins (24–27). Because some membrane-associated enzymes (27–29), including GTPases (30), are known to be sensitive to damage by free radicals, we tested the hypothesis that $\text{A}\beta$ impairs muscarinic cholinergic signal transduction by interfering with receptor-G protein coupling by a free radical-mediated mechanism.

MATERIALS AND METHODS

Neocortical Cell Culture, Experimental Treatments, and Cell Survival. Dissociated cell cultures of embryonic rat (Sprague–Dawley, 18 days of gestation) neocortex were established and maintained as described (31). All experiments were performed in 7–10-day old cultures. Synthetic $\text{A}\beta$ s were purchased from Bachem ($\text{A}\beta_{25-35}$, lots ZL650 and ZJ744; $\text{A}\beta_{1-40}$, lot ZK600), and a control peptide with the same amino acid composition as $\text{A}\beta_{25-35}$, but with a scrambled sequence (20), was used for some experiments. Peptides were stored lyophilized, and stocks were prepared 1–2 hr prior to use by dissolving the peptide at a

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Abbreviations: $\text{A}\beta$, amyloid β -peptide; AD, Alzheimer disease; APP, amyloid precursor protein; DAG, diacylglycerol; G, GTP-binding; IP, inositol triphosphate; PBN, *N*-tert-butyl- α -phenylnitron; PIP_2 , phosphatidylinositol-4,5 bisphosphate; ROS, reactive oxygen species.

‡To whom reprint requests should be addressed at: 211 Sanders–Brown Building, University of Kentucky, Lexington, KY 40536-0230.

concentration of 1 mM in sterile distilled water (vehicle). Carbachol, propyl gallate, vitamin E (α -tocopherol), and glutamate were from Sigma. PBN (*N*-tert-butyl-phenylnitron) was a gift from D. A. Butterfield. Two methods were employed to quantify neuronal survival: counting undamaged neurons in 10 $\times$ microscope fields prior to and at designated time points following exposure to experimental treatments (19, 31); and quantifying lactate dehydrogenase released into the culture medium (18, 31).

Low- K_m GTPase Assay. Cells were homogenized in 500–750 μ l of 50 mM Na⁺/K⁺ phosphate buffer (pH 7.4), 10 mM EDTA, and either 0.1 mM phenylmethylsulfonyl fluoride (PMSF) or 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc SC). Low K_m GTPase activity was determined in duplicate using a protocol similar to previous methods (32–34). Briefly, 25 μ l of crude cell membrane preparation (10–20 μ g of protein) was added to 75 μ l of a reaction mixture consisting of (final concentration): 20 mM Tris-HCl (pH 7.4), 0.3 μ M [γ -³²P]GTP, 5 mM MgCl₂, 0.15 mM ATP, 0.34 mM AppNHp, 6.75 mM phosphocreatine, 81 units of creatine phosphokinase, 0.1 mM EDTA, 0.1 mM EGTA, 1.33 mM DTT, and 100 mM NaCl. Cold GTP (100 μ M) and/or 1 mM carbachol (the concentration of carbachol was at E_{max} for this assay) were added to some samples. After adding protein, samples were vortexed, incubated at 37°C for 10 min, and then 900 μ l of an ice-cold solution of 5% activated charcoal containing phosphoric acid (pH 2.5). Samples were centrifuged at 11,950 $\times g$ for 5 min and a 100 μ l aliquot of the supernatant subsequently removed, mixed with 4 ml of scintillation fluid, and counted with a Wallac model 1410 liquid scintillation counter. Low K_m GTPase activity was calculated by subtracting total activity measured in the presence of 100 μ M unlabeled GTP from basal and carbachol-stimulated GTPase activity.

[³H]QNB Saturation Binding and Carbachol Competition Studies. For saturation binding studies 10–20 μ g of cell sample protein was incubated at 24°C for 50 min in a reaction mixture consisting of 50 mM Tris-HCl, pH 7.5 with 2 mM MgCl₂ and several concentrations of [³H]QNB (45.4 Ci/mmol, DuPont/NEN). Nonspecific binding was defined by inclusion of 10^{–5} M atropine sulfate. The efficiency of carbachol competition for QNB was assessed by adding 1 mM carbachol to a reaction mixture containing a single 5 nM concentration of [³H]QNB. All reactions were terminated by rapid filtration and washing with ice-cold 50 mM Tris-HCl, pH 7.5; radioactivity associated with the filter was counted. Values for B_{max} and K_d were calculated by nonlinear regression using MICROCAL ORIGIN 4.0.

Measurement of IP Accumulation and [Ca²⁺]_i. Levels of radiolabeled IP₁, IP₂, and IP_{3/4} in cells prelabeled with [³H]myoinositol (overnight incubation in the presence of 1 μ Ci/ml; specific activity 20.5 Ci/mmol; 1 Ci = 37 GBq) were quantified as described (35). After exposure for 5 hr to vehicle or A β , cultures were treated with 10 mM LiCl for 10 min before exposure to vehicle or 100 μ M carbachol. Following methanol fixation cells were scraped and lysed, and three volumes of methanol:chloroform (2:1) were added. After a 15-min incubation, chloroform and 0.5 M HCl were added in volumes equal to that of the original lysate. Samples were mixed, centrifuged (1500 $\times g$ for 5 min), and an aliquot (750 μ l) of the aqueous phase was applied to a 2-ml Dowex 1 column pre-equilibrated to the formic acid form. Columns were washed sequentially with 15 ml of 5 mM myoinositol and 5 ml of 5 mM sodium tetraborate/60 mM ammonium formate. They were then eluted into three separate scintillation vials with sequential 3-ml elutions of 0.2, 0.45, and 1 M ammonium formate in 0.1 M formic acid, respectively, and radioactivity was counted. Fluorescence ratio imaging of the Ca²⁺ indicator dye fura-2 was employed to quantify [Ca²⁺]_i in neurons using methods detailed previously (20, 31). Values represent the average [Ca²⁺]_i in the neuronal cell body. Experimental treatments were added to the bathing medium by dilution from 100–500 $\times$ stocks.

RESULTS

A β Impairs Carbachol Stimulation of Low K_m GTPase Activity. Neocortical cell cultures incubated with A β 25–35

FIG. 1. Carbachol stimulation of low K_m GTPase activity is impaired in cultured cortical neurons exposed to A β . (A) Cultures were exposed to 20 μ M A β 25–35 for different time periods and GTPase activity following exposure to 1 mM carbachol was quantified in cell membranes. Values are the mean and SEM (carbachol stimulation expressed as a percent of control values) from five separate sets of experiments. $F = 16.5(4,15)$, $P < 0.001$ by repeated measures analysis of variance (ANOVA). *, $P < 0.05$, **, $P < 0.01$ (Dunnett's test). A β did not affect basal (unstimulated) GTPase activity at any time point. (B) Cultures were incubated for 4 hr with indicated concentrations A β 25–35 and then basal and carbachol-induced GTPase activity in membranes was quantified. Bars are the mean and SEM ($n =$ five experiments). $F = 8.38(4,20)$, $P < 0.001$ by repeated measures ANOVA. *, $P < 0.05$, **, $P < 0.01$, compared with Control value (Dunnett's procedure). A β did not affect basal (unstimulated) GTPase activity. (C) Cultures were exposed for 4 hr to vehicle, 20 μ M A β 1–40 or 20 μ M scrambled A β 25–35. Bars are the mean and range ($n =$ two experiments).

Table 1. Lack of effect of A β on muscarinic cholinergic ligand binding parameters

Parameter	Control	A β
K_d , nM	4.78 $\pm$ 1.4	3.71 $\pm$ 1.5
B_{max} , fmol/mg protein	80 $\pm$ 20	86 $\pm$ 29

The K_d and B_{max} in membranes from control cultures and cultures incubated with A β 25–35 for 4 hr determined from [3 H]QNB saturation binding studies (mean $\pm$ SEM of five separate experiments). Student's *t* test revealed no significant differences for either K_d or B_{max} .

showed a time- and concentration-dependent attenuation of carbachol-stimulated low K_m GTPase activity (Fig. 1*A* and *B*). There was no significant reduction in basal GTPase activity. Incubation with 20 μ M A β 25–35 reduced carbachol stimulation after 20 min and produced a maximum reduction after 1 hr. A similar time profile was apparent when 50 μ M was used (data not shown). Attenuation of the carbachol stimulation of low K_m GTPase activity could be detected even at nanomolar concentrations of A β 25–35. Ten nanomolar A β reduced GTPase activity to 60% of the control value. The specificity of this effect was confirmed by performing low K_m GTPase assays on cultures incubated with A β 1–40 and with an A β 25–35 peptide with a scrambled amino acid sequence (Fig. 1*C*). While A β 1–40 produced a highly significant decrease in carbachol-induced GTPase activity, the scrambled peptide had no effect.

To assess whether the decrease in carbachol stimulation of low K_m GTPase activity caused by A β was due to an effect on ligand binding to receptors, we determined B_{max} and K_d from the results of saturation binding assays with [3 H]QNB (Table 1). There was no difference between control cells and cells incubated with A β 25–35 for either parameter. There was no difference between control and A β -exposed cultures in the efficacy of 1 mM carbachol (the concentration used for low K_m GTPase assays) displacement of [3 H]QNB binding. In control cells, as well as those exposed to A β 25–35 (50 μ M for 4 hr), this equaled 100%.

Because A β can be neurotoxic, we assessed the effects of increasing concentrations of A β on neuronal survival using both cell counting and lactate dehydrogenase release assays. A β 25–35 at concentrations from 10 nM to 20 μ M was not neurotoxic during a 5-hr exposure period (Fig. 2), whereas calcium ionophore A23187 produced marked neuronal degeneration during the same exposure period (Fig. 2*B*). During a 24-hr exposure period no significant reduction in neuronal survival was seen with A β concentrations of 10 and 100 nM, whereas concentrations of 1 μ M and above progressively decreased neuronal survival (data not shown; ref. 20).

Carbachol-Induced IP Release Is Suppressed in Cortical Cells Exposed to A β . A 20-min stimulation of neocortical cultures with

FIG. 2. Levels of A β that impair muscarinic receptor signaling are subtoxic. Cultures were exposed to the indicated concentrations of A β 25–35 for 5 hr and neuronal survival was quantified by cell counts (*A*) or measurement of lactate dehydrogenase levels in the culture medium (*B*). Values are the mean and SEM of determinations made in three separate cultures. *, $P < 0.001$ compared with each of the other values. ANOVA with Scheffe's post-hoc tests.

carbachol (100 μ M) typically evoked a 3- to 5-fold elevation in the accumulation of total IP species (Fig. 3). After a 5-hr exposure to 20 μ M A β 25–35, neocortical cultures showed a reduced response to carbachol for all IP species. The largest effect of A β exposure was seen in IP $_2$, IP $_3$, and IP $_4$ species; in fact, the stimulation by carbachol of IP $_3$ and IP $_4$ levels was completely prevented by A β .

FIG. 3. Carbachol-induced accumulation of IP is attenuated in cortical cells pretreated with A β . Following incorporation of [3 H]myo-inositol cells were exposed to vehicle or 20 μ M A β 25–35 for 5 hr. Cells were then exposed to either vehicle or carbachol for 20 min in the presence of lithium (groups not receiving carbachol were exposed to lithium for equivalent times). Levels of IP, IP $_2$, and IP $_3/4$ were determined. Values are the mean $\pm$ SEM of triplicate cultures. *, $P < 0.005$, **, $P < 0.05$, compared with carbachol-treated cultures not exposed to A β (Carb).

FIG. 4. A β abrogates carbachol-induced release of calcium from intracellular stores in neocortical neurons. (A) Cultures were incubated in medium lacking added Ca^{2+} and containing 1 mM EGTA (0[Ca]_o). The $[Ca^{2+}]_i$ was monitored at 7-sec intervals before and after exposure to 100 μ M carbachol. The traces represent the average $[Ca^{2+}]_i$ in from 12 to 22 neurons. (B) Parallel cultures were preincubated for 3 hr in the presence of 50 μ M A β 25–35 or vehicle (control).

Pretreatment with A β did not block IP responses to glutamate and aluminum fluoride, indicating that A β did not cause a generalized impairment of phospholipase C or reduction of phosphatidylinositol pools (data not shown).

Carbachol-Induced Calcium Release Is Suppressed in Neurons Exposed to A β . Exposure of control cultures to carbachol resulted in a transient, approximately 2-fold, elevation of $[Ca^{2+}]_i$. The carbachol-induced $[Ca^{2+}]_i$ elevation was due to Ca^{2+} release from intracellular stores because the response was observed in Ca^{2+} -free medium (Fig. 4A). When cultures were pretreated with 50 μ M A β 25–35 for 3–4 hr prior to $[Ca^{2+}]_i$ imaging, there was a modest ($\approx$ 50 nM) increase in rest $[Ca^{2+}]_i$, and the carbachol-induced $[Ca^{2+}]_i$ response was markedly attenuated or completely eliminated (Fig. 4B). The ability of carbachol to induce $[Ca^{2+}]_i$ elevation was progressively reduced in a concentration-dependent manner in neurons pretreated with A β ; concentrations of 100 nM or greater produced statistically significant effects (Fig. 4C).

Antioxidants Attenuate A β -Induced Impairment of Carbachol-Induced GTPase Activity and Ca^{2+} Release. In light of data implicating reactive oxygen species (ROS) generation in the mechanism of A β toxicity (23–27), we tested the hypothesis that ROS mediated loss of responsiveness to carbachol induced by A β . The chain-breaking inhibitor of lipid peroxidation vitamin E (α -tocopherol) and propyl gallate (inhibitor of oxidation induced by superoxide radicals and lipid peroxidation) were employed (36). For determination of low K_m GTPase activity cultures were pretreated for 1–20 hr with vitamin E (50 μ g/ml) and 1 hr with 10–50 μ M propyl gallate, followed by 4 hr of exposure to 20–50 μ M A β 25–35. Both vitamin E and propyl gallate significantly attenuated A β -induced disruption of carbachol-stimulated GTPase activity (Fig. 5A). Basal low K_m GTPase activity was not altered by the antioxidants alone (data not shown). FeSO₄ induced a time-dependent reduction in carbachol-induced GTPase activity that paralleled that observed with A β (Fig. 5B), strengthening the hypothesis that ROS mediated A β -induced impairment of muscarinic signaling. Pretreatment of cultures with vitamin E, propyl gallate, or the spin-trapping antioxidant PBN (37) prevented the loss of $[Ca^{2+}]_i$ response to carbachol in neurons exposed to A β (Fig. 6).

DISCUSSION

A β disrupted muscarinic cholinergic agonist stimulation of receptor-G protein coupling linked to inositol phospholipid hydrolysis without producing neuronal cell injury or death. The effect of A β on carbachol stimulation of low K_m GTPase activity was specific because: (i) similar effects were produced by incubation with full-length (A β 1–40) peptide and not scrambled A β 25–35; (ii) the effect was time-dependent with maximal reduction after 1 hr, and concentration-dependent in a physiologically relevant range; (iii) there was no change in B_{max} and K_d or in efficacy of carbachol competition measured in [³H]QNB saturation and competition binding assays eliminating the possibility that A β damages muscarinic receptors; (iv) disruption of carbachol-induced GTPase activity occurred with subtoxic concentrations of A β and at time points many hours prior to degeneration in neurons exposed to toxic concentrations of A β . Moreover, two downstream events in the muscarinic cholinergic receptor-mediated signal transduction cascade, IP accumulation

The $[Ca^{2+}]_i$ was then monitored prior to and following exposure to 100 μ M carbachol. Values are the mean of 10–14 neurons. Similar results were obtained in three additional experiments. (C) Cultures were exposed to the indicated concentrations of A β 25–35 and then exposed to vehicle (leftmost value) or 100 μ M carbachol. Values represent the peak $[Ca^{2+}]_i$ (and SEM) following exposure to vehicle or carbachol (n = 16–24 neurons in three separate cultures). *, P < 0.01 compared with 0 A β + carbachol value.

FIG. 5. Free radicals mediate disruption of carbachol-induced GTPase activity by A β . (A) Cultures were pretreated for 20 hr with vehicle or 50 μ g/ml vitamin E, or for 1 hr with 50 μ M propyl gallate, and then exposed to vehicle (Control) or 20 μ M A β 25-35 for 4 hr. Values are the mean and SEM ($n = 6$ experiments). $F = 6.11(3,14)$, $P < 0.01$ determined by one-way ANOVA. **, $P < 0.01$ compared with control value (Dunnett's procedure). (B) Cultures were exposed to 5 μ M FeSO₄ for the indicated time periods and then basal and carbachol stimulated low K_m GTPase activity in membranes was quantified. For A β -treated cultures values are the mean and SEM ($n = 3$ separate cultures). For FeSO₄-treated cultures values are the mean of duplicate determinations.

and calcium release, were also reduced by A β . A β therefore appears to have a specific effect on the efficiency of G protein coupling to muscarinic receptors.

Our findings in this model system are consistent with those described in studies of postmortem brain tissue obtained from AD patients that have shown evidence of a reduction in the proportion of M1 receptors present in the high-affinity (G protein-coupled) state (4-7). The abnormalities in receptor-G protein coupling in AD do not appear to be related to a decrease in muscarinic receptors (38, 39) or G proteins (40, 41). The defect(s) underlying such changes in receptor-G protein coupling are unknown but could include specific alterations in G protein subunits. AD-related defects in guanine nucleotide exchange may not be limited to G proteins coupled to heterotrimeric G proteins as suggested by the report of a reduction in tubulin-mediated transfer of guanine nucleotides to G proteins in AD (42). Alterations in G protein coupling could also result from changes in the composition and biophysical properties of the plasma membrane that could affect G protein conformation or motional properties (43). Indeed, Muller *et al.* (44) reported that A β produced a decrease in membrane fluidity in neurons that was detectable at nanomolar concentrations of the peptide.

FIG. 6. Antioxidants prevent A β -induced impairment of calcium responses to carbachol. (A) Parallel cultures were pretreated for 16 hr with 50 μ g/ml vitamin E or vehicle (control and A β) and then incubated for 3 hr in the presence of 50 μ M A β 25-35 or vehicle (control). The [Ca²⁺]_i was then monitored before and after exposure to 100 μ M carbachol. Values represent the mean of 12-18 neurons in a representative experiment. (B) Cultures were pretreated for 16 hr with 50 μ g/ml vitamin E or vehicle (Control), or 1 hr with 50 μ M PBN or 50 μ M propyl gallate. Cultures were then exposed for 3 hr to A β and [Ca²⁺]_i was monitored before and after exposure to 100 μ M carbachol. Values represent the peak [Ca²⁺]_i (≈ 30 sec following exposure to carbachol; mean and SEM of 9-18 neurons). *, $P < 0.001$ compared with values for control, VitE+A β and PBN+A β . **, $P < 0.05$ compared with A β value, and $P < 0.02$ compared with control value. ANOVA with Scheffe's post-hoc tests.

Present and previous data indicate that the mechanism of A β neurotoxicity involves induction of ROS. A β induced accumulation of peroxides in cultured neurons (23, 25) and lipid peroxidation in cultured neurons (25) and gerbil brain synaptosomes (26). A major target of ROS is membrane phospholipids containing unsaturated fatty acyl chains. Free (radicals) induced by A β in fact appear to localize in the mid bilayer region where fatty

acyl chains are enriched (26, 27). Because plasma membrane enzymes have been shown to have different susceptibilities to oxidative damage (18), it will be important to determine whether different GTP-binding proteins are impaired by free radicals. Because oxidative stress is a prominent feature of many different neurodegenerative conditions (45), an array of signaling pathways involving G proteins may be adversely affected.

Previous studies have shown that nanomolar concentrations of A β can alter physiological properties of cells. For example, low concentrations of A β can alter K⁺ channels in cultured human fibroblasts (46) and can induce tyrosine phosphorylation of several proteins in culture (47). However, in the latter studies the roles of peptide aggregation and free radicals were not assessed, and a recent study showed that induction of tyrosine phosphorylation of focal adhesion kinase in cultured neuroblastoma cells was mediated by an aggregation-dependent mechanism that was likely receptor-independent (48). The present findings show that subtoxic levels of A β impair muscarinic signal transduction, and that toxic levels of A β impair this signaling pathway many hours prior to cell degeneration. These data suggest that disruption of cholinergic transmission may therefore be an early event in AD. Whether other G protein-linked signaling pathways are similarly altered by A β remains to be established. In ongoing studies we found that A β , at concentrations up to 50 μ M, does not impair coupling of metabotropic glutamate receptors to their GTP-binding proteins (M.P.M. and E.M.B., data not published). On the other hand, A β did alter calcium responses to thrombin (thrombin receptors activate a G_q protein linked to phospholipase C) in cultured human neural cells; those effects of A β were blocked by antioxidants (49). Because neurons employ a myriad of receptors linked to G proteins, it seems likely that some of these pathways will be adversely affected by A β and oxidative processes.

In addition to contributing to functional aspects of the cognitive impairments seen in AD, A β -induced alterations in muscarinic cholinergic receptor-G protein coupling may affect the neurodegenerative process itself. Muscarinic cholinergic receptor mediated pathways may be involved in promoting cleavage of β APP by α -secretase to release secreted forms of APP (50, 51); this secretory pathway precludes deposition of amyloidogenic A β . In contrast to A β , secreted APPs have neurotrophic activities and can protect neurons against excitotoxicity and oxidative insults including A β toxicity (23, 52). A decrease in receptor-G protein coupling induced by A β would therefore be expected to lead to a "vicious cycle" characterized by A β deposition and reduction in levels of trophic secreted APPs. In addition, because activation of muscarinic receptors may increase expression of neurotrophic factors (53, 54), A β -induced impairment of cholinergic transmission may reduce levels of trophic support. Finally, our findings may help explain the relative ineffectiveness of agents such as Tacrine that can only enhance cholinergic neurotransmission when the postsynaptic receptor mediated signal transduction pathway is intact. They also provide a rationale for investigating the efficacy of antioxidants in the treatment of AD.

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