Release of Alzheimer Amyloid Precursor Derivatives Stimulated by Activation of Muscarinic Acetylcholine Receptors

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types that normally undergo apoptosis (20).

The finding that Bcl-2 is an inner mitochon-
drial membrane protein (11) raises the possi-
blility that mitochondria may play an im-
portant role in apoptosis. Understanding the
mode of action of Bcl-2 might provide
insights into the nature of degenerative diseases (21).

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9. Bcl-2 DNA insert from pB4 (Y. Tsujimoto and C. M.
   (1986) containing the human bcl-2 coding re-
   gion) was excised with Eco RI, blunt-ended, and
   subcloned into the blunt-ended Sph I site of pNS-LacZ
   [S. Forss-Petter et al., Nature 356, 187 (1992)]
   (pNS-LacZ contains a 1.8-kb 5' flanking
   DNA sequence from the rat neuron-specific endo-
   lase (NSE) gene fused to the LacZ gene). A 2.7-kb
   fragment containing the 1.8-kb 5' flanking
   region of the NSE gene fused to bcl-2 was
   excised from this plasmid with Sac I (partial
   digestion followed by blunt ending) and Hind III.
   This fragment was finally subcloned in front of
   the SV40 T intron and polyadenylation signal
   already inserted into the Bluescript cloning vec-
   tor pSK+ [Stratagene, San Diego, CA]. This
   plasmid was called pB4.


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16. The Bcl-2 protein was detected by immunocyto-
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    as well as in motoneurons from a 10-week-old human
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22. Sympathetic neurons from superior cervical gan-
    glia were cultured as previously described [E.
    Hawrot and P. H. Patterson, Methods Enzymol. 53,
    574 (1979)]. Circular plasmids were dissolved in
    tris-EDTA buffer at a concentration of 0.1 mg/
    ml. Approximately 500 DNA molecules were intro-
    duced per cell with a low-pressure microinjection
    system (automatic injector InJet c-Matic, Geneva)
    to ensure high neuronal survival [I. García et al.,
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23. Mouse monoclonal antibodies used in this study
    were Bcl-2-100 and Bcl-2-124 (10). After micro-
    injection, neurons were kept in N2RgF medium
    for 3 days before measuring Bcl-2 immunoreac-
    tivity. Neurons were fixed in 4% paraformalde-
    hyde in phosphate-buffered saline (PBS) for 20
    min at room temperature, permeabilized in 0.1%-
    Triton X-100 in PBS, and incubated with anti-
    Bcl-2 and then rhodamine-conjugated rat anti-

24. Living cells were incubated with rhodamine 123
    (Sigma) (1 µg/ml PBS for 30 min at 37°C, rinsed
    three times with PBS, and fixed in 4% paraformal-
    dehyde and 0.5% glutaraldehyde.

25. We thank S. Forss-Petter for neuron-specific
    enolase promoter; D. Y. Mason for Bcl-2 mono-
    clonal antibodies; P. Schwartz (Zeiss, Zurich) for
    the confocal microscopy; R. Zuffery for S4V T
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Release of Alzheimer Amyloid Precursor
Derivatives Stimulated by Activation of Muscarinic Acetylcholine Receptors

Roger M. Nitsch, Barbara E. Slack, Richard J. Wurtman, John H. Growdon

Altered processing of the amyloid precursor protein (APP) is a central event in the formation of amyloid deposits in the brains of individuals with Alzheimer’s disease. To investigate whether cellular APP processing is controlled by cell-surface neurotransmitter receptors, human embryonic kidney (293) cell lines were transfected with the genes for human brain muscarinic acetylcholine receptors. Stimulation of m1 and m3 receptor subtypes with carbachol increased the basal release of APP derivatives within minutes of treatment, indicating that preexisting APP is released in response to receptor activation. Receptor-activated APP release was blocked by staurosporine, suggesting that protein kinases mediate neurotransmitter receptor–controlled APP processing.

The deposition of extracellular amyloid in brain parenchyma is characteristic of Alzheimer’s disease pathology (1). Amyloid deposits consist of aggregates of a 39–
43-amino acid peptide termed βA4 (2), which is an abnormal cleavage product of a larger APP (3). Amyloid precursor protein is an integral membrane glycoprotein, exist-

ing as several distinct forms derived from alternative mRNA splicing (4). Water-soluble APP fragments lacking the COOH-terminal have been detected in condi-
tioned cell culture media and in human cerebrospinal fluid (5), indicating that APP is a secretory protein. Normal secretion of water-soluble NH2-terminal APP deriva-
tives involves cleavage of full-length APP at an extracellular site located close to the transmembrane domain and within the βA4 domain (6). This cleavage event pre-
sumably precludes the formation of amy-
loidogenic APP fragments. Other than be-
coming processed by secretion, APP can be processed by an internal lysosomal pathway (7) that may generate amyloidogenic cleavage products (8). It is therefore likely that aberrations in the regulation of APP pro-
cessing pathways contribute to amyloid for-
mation. The mechanisms regulating cellu-
lar APP processing, however, are un-
known.

To investigate whether APP processing is controlled by activation of cell-surface neurotransmitter receptors, we used cul-
tured human 293 cell lines transfected with and stably expressing the genes for the human brain muscarinic acetylcholine recep-
tor (mAChR) subtypes m1, m2, m3, or m4 (9). The 293 cells express full-length human APP, secrete a large NH2-terminal APP derivative, and retain an 11.5-kD COOH-terminal APP fragment (10), indic-

ative of normal APP cleavage and secre-
tion by this cell line. Activation of muscarinic neurotransmitter receptors by the stable cholinergic agonist carbachol in 293 cells expressing the genes for the m1 (Fig. 1A) and m3 (Fig. 1B) mACHR subtypes potently stimulated the release of water-soluble APP fragments into the cell culture media within 30 min (11). Densitometric analysis of the Western blots (12) indicated that receptor activation with carbachol increased the basal release of APP derivatives 3.9-fold (range 2.75 to 6.0, n = 7) in the m1-transfected cells and 5.4-fold (range 4.1 to 6.7, n = 7) in the m3-transfected cells (Fig. 1D). These results show that cellular release of APP derivatives can be controlled by cell surface neurotransmitter receptor activity. Basal release of APP fragments from m2 mACHR- or m4 mACHR-transfected cells and from wild-type cell lines was not stimulated by carbachol (Fig. 1, C and D). These results indicate that neurotransmitter control of APP processing is receptor subtype-specific, presumably owing to the differential coupling of the receptor subtypes to distinct second messenger systems: activation of the phospholipase C–linked mACHR subtypes m1 and m3 (9, 13) stimulated APP release, whereas the adenylyl cyclase–linked subtypes m2 and m4 (9) did not mediate this response (Fig. 1D).

Receptor-activated APP release by both m1- (Fig. 1A) and m3- (Fig. 1B) transfected cell lines was blocked by the muscarinic antagonist atropine, indicating a specific agonist-receptor interaction (Fig. 1E). Stimulation of m1 and m3 receptors activates protein kinase C (PKC) by increasing diacylglycerol formation (14), which, along with inositol phosphates, is a product of phosphatidylinositol hydrolysis (9, 13). Receptor-coupled release of APP derivatives may thus be mediated by diacylglycerol-induced PKC activation or by an interaction of diacylglycerol and calcium released from internal pools by inositol trisphosphate (15). Protein kinase C activation by phorbol esters has been shown to increase both the release of NH2-terminal APP derivatives and the abundance of cell-associated COOH-terminal APP cleavage products in PC-12 cells and to phosphorylate APP at Ser635 in semi-intact PC-12 cells (16). Furthermore, purified PKC can directly phosphorylate a synthetic COOH-terminal APP fragment (17). These data, as well as our results, however, do not rule out the possibility that phosphorylation of other proteins (for example, proteases) is involved in the regulation of APP release. A causal relation between phosphorylation of APP and its cleavage has not been established. Furthermore, our observations do not exclude the possibility that other receptor species linked to phosphatidylinositol turnover, and thus to receptor-coupled generation of diacylglycerol with subsequent activation of PKC, may also regulate APP release. In four independent experiments, bradykinin caused a rapid increase in the release of APP fragments from differentiated PC-12 cells (18). Like the muscarinic m1 and m3 receptors, the bradykinin receptor is also linked to activation of phospholipase C and PKC. Release of APP derivatives was not stimulated by increased intracellular calcium levels alone, as indicated by the failure of the calcium ionophore A23187 to mimic the receptor-mediated stimulation of basal release (Fig. 1, A, B, and E).

The effect of both m1 and m3 mACHR stimulation on the release of APP derivatives was rapid: half-maximal stimulation was reached within 5 to 10 min, and maximum stimulation was attained within 15 to 30 min (Fig. 2, A and B). These time-
creted APP fragments were cleavage products of the parent protein lacking the COOH-terminus, we used a polyclonal antisem against the COOH-terminus (20). Western blot analysis of cell-associated APP in both m1 mAChR- and m3 mAChR-transfected cell lines with the use of this antisem revealed the expected pattern of APP and APP fragments (Fig. 3B), which was similar to that described in human brain cortex (20). This antisem did not detect any APP fragments in conditioned media obtained from stimulated or unstimulated m1- and m3-transfected cell lines (Fig. 3B), indicating that the secreted APP fragments are indeed APP cleavage products lacking the COOH-terminus. These data might imply that stimulation of the secretory pathway by receptor activation is associated with decreased endosomal-lysosomal generation of amyloidogenic APP fragments (8).

It is important to note that the transfected cell lines used in these experiments express larger than normal numbers of muscarinic receptors. However, other studies in our laboratory indicate that stimulation of endogenous neurotransmitter receptors in nontransfected, differentiated PC-12 cells also increases the release of APP fragments, indicating that physiologic receptor levels are sufficient to mediate this response (18). We have also observed that electrical stimulation of rat hippocampal slices in vitro evoked a frequency-dependent, tetrodotoxin-sensitive increase in the release of APP fragments (18).

In summary, we report a cell-surface neurotransmitter receptor-mediated mechanism for the stimulation of rapid release of soluble NH2-terminal APP derivatives. This mechanism presumably is mediated by receptor-coupled phosphatidylinositol turnover in the same cell lines (13). Western blots of cell-associated APP (19) with the monoclonal antibody 22C11 showed that stimulation with carbachol decreased the abundance of the 141-kD APP, whereas the amount of 115-kD APP remained unaffected (Fig. 3A). The concomitant carbachol-induced increase of a 128-kD APP fragment secreted into the culture medium (Fig. 3A) suggests that the secreted 128-kD protein is a cleavage product of the cell-associated 141-kD APP.

In order to investigate whether the secreted APP fragments were cleavage products of the parent protein lacking the COOH-terminus, we used a polyclonal antisem against the COOH-terminus (20). Western blot analysis of cell-associated APP in both m1 mAChR- and m3 mAChR-transfected cell lines with the use of this antisem revealed the expected pattern of APP and APP fragments (Fig. 3B), which was similar to that described in human brain cortex (20). This antisem did not detect any APP fragments in conditioned media obtained from stimulated or unstimulated m1- and m3-transfected cell lines (Fig. 3B), indicating that the secreted APP fragments are indeed APP cleavage products lacking the COOH-terminus. These data might imply that stimulation of the secretory pathway by receptor activation is associated with decreased endosomal-lysosomal generation of amyloidogenic APP fragments (8).

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In order to investigate whether the

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11. Human 293 cell lines were transfected with the human genes for the m1, m2, m4, and mAChR subtypes as well as for neomycin resistance as described (9) and grown in Dulbecco's minimum essential medium–F12 medium containing 10% fetal bovine serum (fascia) in the presence of the neomycin analog G418 (0.5 mg/ml) (Geneticin, Gibco). Nontransfected human 293 cells were obtained from the American Type Culture Collection (CRT 1573) and maintained in the absence of G418. The cell lines expressed a specific number of receptors per cell: m1, 350,000; m2, 120,000; m3, 200,000; m4, 50,000, and wild-type, <300. Confluent cell cultures on poly-lysine-coated (relative molecular mass >300,000) 0.1 mg per dish culture dishes were incubated with water-free N-2 medium (Gibco) and incubated for 30 or 60 min in serum-free N-2 with or without 1 nm carbachol (Sigma), 10 nM atropine (Sigma), 1 μM staurosporine (Sigma), or 10 μM A23187 (Sigma), in the absence of G418. Conditioned media were centrifuged at 10,000 g (4°C, 5 min), desalted on Sephadex G-25 columns (Pharmacia) with water as eluent, lyophilized, and reconstituted typically in 150 μl of SDS loading buffer. Total cell protein was measured with the biocinchoninic acid assay (Pierce). Cell proteins were not altered by any of the above treatments. Reconstituted culture media proteins corresponding to 150 μg of total cell protein were separated by SDS-polyacrylamide gel electrophoresis on 12% gels, and Western blots were done with the monoclonal antibody, 22C11 directed against a purified full-

length APP fusion protein (5). Molecular weight standards were obtained from Amersham.

We compared immunoreactive bands densitometrically with the use of an LKB Ultrascan laser scanner. Scanning parameters included 40-μm vertical scanning intervals at a total slit width of 2.4 mm (3 × 0.8 mm), and automatic subtraction of the absorbance offset, which was determined for each blot individually. Measurements were performed in the linear range, which we determined using dilution curves of cell protein extracts. Results were normalized to basal APP release from vehicle-treated controls, determined within the same blot. Experiments were done with duplicate or triplicate cell culture dishes and repeated five to seven times for statistical analysis with Mann-Whitney rank sum tests or analysis of variance and post hoc Scheffé tests as indicated.

Acetylcholine Receptor Channel Structure Probed in Cysteine-Substitution Mutants

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In order to understand the structural bases of ion conduction, ion selectivity, and gating in the nicotinic acetylcholine receptor, mutagenesis and covalent modification were combined to identify the amino acid residues that line the channel. The side chains of alternate residues—Ser248, Leu256, Ser252, and Thr254—in M2, a membrane-spanning segment of the α subunit, are exposed in the closed channel. Thus α248-254 probably forms a β strand, and the gate is closer to the cytoplasmic end of the channel than any of these residues. On channel opening, Leu256 is also exposed. These results lead to a revised view of the closed and open channel structures.

Nicotinic receptors transduce the binding of acetylcholine (ACh) into the opening of a cation-conducting channel (1). The five subunits (2) of these receptors form a pseudo-symmetric (3) ring around a central channel (4). Each subunit contains four membrane-spanning segments, M1 through M4 (3, 5); both the NH2-terminals and the COOH-terminals of each subunit are extracellular (6, 7). The cation-conducting channel must be formed by residues of the membrane-spanning segments. Mutagenesis of charged residues flanking M2 (Fig. 1) in all four subunits demonstrated the influence of these residues on ion conduction (7, 8) and selectivity (9). Within M2, mutations of residues homologous to Thr244 of the Torpedo α subunit altered cation selectivity (10, 11), mutations of residues homologous to Ser248 and Ser252 of the α subunit influenced the binding of the channel blocker QX-222 (12), and mutation of a residue homologous to Torpedo α subunit Leu251 decreased $K_{app}$ (concentration of agonist eliciting a half-maximal response), revealed a new high-conductance state, decreased the rate of desensitization, and abolished block by QX-222 (13). In a different approach, Torpedo α subunit Ser248 (14, 15) and the homologous residues in the β (14, 16), γ (17), and δ subunits (18) were photolabeled by noncompetitive inhibitors that are believed to bind within the channel. Other M2 residues photolabeled were the residues in the β (16) and γ subunits (17) homologous to α subunit Leu251 and a residue in the γ subunit (17) homologous to α subunit Thr244. A residue flanking M2, α subunit Glu256, was also labeled (19).

We assume (i) that residues lining the cation-conducting pathway of the receptor constitute a portion of the water-accessible surface of the protein; (ii) that these residues are accessible to sufficiently small, charged reagents, at least in the open state of the channel; (iii) that the addition of a charged group to a channel-lining residue would alter ion conduction; and (iv) that residues in the membrane-spanning segment but not exposed in the channel are inaccessible to charged, lipophobic reagents. We mutated consecutive residues in M2 of the mouse muscle α subunit to cysteine and expressed the mutant receptors in Xenopus oocytes. We determined the susceptibility of these mutant receptors to irreversible channel-block by small, charged, sulfhydryl-specific reagents. The technique of substitution of residues with cysteine and chemical modification of the new sulfhydryls has been used in structural studies of cysteine residues (20, colin (21), and bacteriorhodopsin (22).

The small, charged, sulfhydryl-specific reagents, which we synthesized, are the three methanethiosulfonate (MTS) derivatives, CH3SO2SCH2CH2NH2+ (MTSEA), CH3SO2SCH2CH2NMe3+ (MTSET), and CH3SO2SCH2CH2SO3− (MTSES) (23). These MTS derivatives are at least 2500 times as soluble in water as in n-octanol (23). Although they differ somewhat in size, each MTS derivative fits into a cylinder about 0.6 nm in diameter and about 1 nm in length; hence each should fit into the open channel of the ACh receptor, which conducts organic cations up to 0.65 nm in diameter (24). Nevertheless, these reagents might not react even with a solvent-exposed side chain due to steric hindrance because their lengths are greater than the minimal diameter of the channel. These reagents specifically add the $-\text{SCH2CH2R}$ moiety to reduced sulfhydryls to form mixed disulfides (25). The MTS derivatives were added to the oocytes at concentrations of 1 mM for MTSET, 2.5 mM for MTSEA, and 10 mM for MTSES in order to compensate for their different reactivities with non-protein sulfhydryls (23).

We mutated one at a time nine consecutive residues in M2 (240 to 254) of the mouse muscle α subunit to cysteine (Fig. 1) and injected the mutant α subunit mRNA, together with wild-type β, γ, and δ subunit mRNAs, into oocytes (26). One to 3 days

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