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 mitochondrial membrane protein (*11*) raises the possibility that mitochondria may play an important role in apoptosis. Understanding the mode of action of Bcl-2 might provide insights into the nature of degenerative diseases (*21*).

REFERENCES AND NOTES

1. A. A. Glücksmann, *Biol. Rev. Cambridge Philos. Soc.* **26**, 59 (1950); R. E. Ellis, J. Yuan, H. R. Horvitz, *Annu. Rev. Cell Biol.* **7**, 663 (1991); M. C. Raff, *Nature* **356**, 397 (1992).
2. J. J. Cohen and R. C. Duke, *J. Immunol.* **132**, 38 (1984); L. M. Schwartz, L. Kosz, B. K. Kay, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6594 (1990).
3. D. Purves, *Body and Brain: A Trophic Theory of Neural Connections* (Harvard Univ. Press, Cambridge, 1988); Y.-A. Barde, *Neuron* **2**, 1525 (1989); R. W. Oppenheim, *Annu. Rev. Neurosci.* **14**, 453 (1991).
4. Y. Tsujimoto, L. R. Finger, J. Yunis, P. C. Nowell, C. M. Croce, *Science* **226**, 1097 (1984).
5. D. L. Vaux, S. Cory, J. M. Adams, *Nature* **335**, 440 (1988); G. Nuñez *et al.*, *J. Immunol.* **144**, 3602 (1990).
6. C. L. Sentman, J. R. Shutter, D. Hockenbery, O. Kanagawa, S. J. Korsmeyer, *Cell* **67**, 879 (1991); A. Strasser, A. W. Harris, S. Cory, *ibid.*, p. 889.
7. D. P. Martin *et al.*, *J. Cell Biol.* **106**, 829 (1988).
8. A. Batistatou and L. A. Greene, *ibid.* **115**, 461 (1991).
9. The DNA insert from pB4 [Y. Tsujimoto and C. M. Croce, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5214 (1986)] containing the human *bcl-2* coding region was excised with Eco RI, blunt-ended, and subcloned into the blunt-ended Sph I site of pNSE-LacZ [S. Forss-Peter *et al.*, *Neuron* **5**, 187 (1990)] [pNSE-LacZ contains a 1.8-kb 5′ flanking DNA sequence from the rat neuron-specific enolase (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 vector pSK+ (Stratagene, San Diego, CA). This construction was called EB-2.
10. F. Pezzella *et al.*, *Am. J. Pathol.* **137**, 225 (1990).
11. D. Hockenbery, G. Nuñez, C. Milliman, R. D. Schreiber, S. J. Korsmeyer, *Nature* **348**, 334 (1990).
12. L. V. Johnson, M. L. Walsh, L. B. Chen, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 990 (1980).
13. K. J. Lazarus, R. A. Bradshaw, N. R. West, R. P. Bunge, *Brain Res.* **113**, 159 (1976).
14. N. P. Singh and R. E. Stephens, *Stain Technol.* **61**, 315 (1986); J.-C. Martinou, A. Le Van Thai, G. Cassar, F. Roubinet, M. J. Weber, *J. Neurosci.* **9**, 3645 (1989).
15. M. Negrini, E. Silini, C. Kozak, Y. Tsujimoto, C. M. Croce, *Cell* **49**, 455 (1987); D. M. Hockenbery, M. Zutter, W. Hickey, M. Nahm, S. J. Korsmeyer, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6961 (1991).
16. The Bcl-2 protein was detected by immunocytochemistry in sympathetic and sensory neurons as well as in motoneurons from a 10-week-old human fetus (J.-C. Martinou, unpublished results).
17. G. Pilar and L. Landmesser, *J. Cell Biol.* **68**, 339 (1976); I. W. Chu-Wang and R. W. Oppenheim, *J. Comp. Neurol.* **177**, 33 (1978).
18. A. H. Wyllie, R. G. Morris, A. L. Smith, D. Dunlop, *J. Pathol.* **142**, 67 (1984); T. Koike, D. P. Martin, E. M. Johnson, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6421 (1989).
19. V. G. Borzillo, K. Endo, Y. Tsujimoti, *Oncogene* **7**, 869 (1992).
20. M. O. Hengartner, R. E. Ellis, H. R. Horvitz, *Nature* **356**, 494 (1992).
21. D. C. Wallace, *Science* **256**, 628 (1992).
22. Sympathetic neurons from superior cervical ganglia 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 introduced per cell with a low-pressure microinjection system (automatic injector Inject+Matic, Geneva) to ensure high neuronal survival [I. Garcia *et al.*, *Mol. Cell Biol.* **51**, 294 (1986)].
23. Mouse monoclonal antibodies used in this study were Bcl-2-100 and Bcl-2-124 (*10*). After microinjection, neurons were kept in NGF-rich medium for 3 days before measuring Bcl-2 immunoreactivity. Neurons were fixed in 4% paraformaldehyde 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-bodies to mouse immunoglobulin G (Boehringer).
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% paraformaldehyde and 0.5% glutaraldehyde.

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

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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– to 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, existing as several distinct forms derived from alternative mRNA splicing (*4*). Water-soluble APP fragments lacking the COOH-terminus have been detected in conditioned cell culture media and in human cerebrospinal fluid (*5*), indicating that APP is a secretory protein. Normal secretion of water-soluble NH_2-terminal APP derivatives 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 presumably precludes the formation of amyloidogenic APP fragments. Other than being 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 processing pathways contribute to amyloid formation. The mechanisms regulating cellular APP processing, however, are unknown.

To investigate whether APP processing is controlled by activation of cell-surface neurotransmitter receptors, we used cultured human 293 cell lines transfected with and stably expressing the genes for the human brain muscarinic acetylcholine receptor (mAChR) subtypes m1, m2, m3, or m4 (*9*). The 293 cells express full-length human APP, secrete a large NH_2-terminal APP derivative, and retain an 11.5-kD COOH-terminal APP fragment (*10*), indicative of normal APP cleavage and secre-

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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).

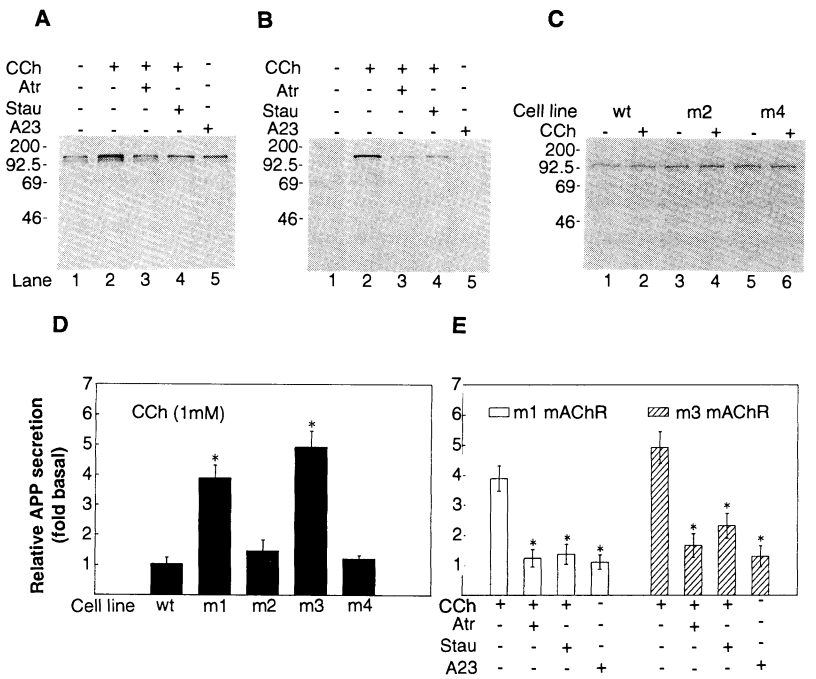

Fig. 1. Western blots of APP derivatives secreted by human 293 cell lines transfected with the genes for the human brain mAChR subtypes m1 (**A**), m3 (**B**), and m2, m4, and nontransfected wild-type 293 cells (**C**) with the use of the monoclonal antibody 22C11 (*11*). CCh, carbachol; Atr, atropine; Stau, staurosporine; A23, calcium ionophore A23187; and wt, wild type. Molecular size markers are shown at the left (in kilodaltons). Densitometric analysis of secreted APP fragments after stimulation with carbachol [(**D**) $^*P < 0.01$ versus basal APP release, Mann-Whitney rank sum tests] and under various other conditions [(**E**) $^*P < 0.01$ versus CCh-induced APP release, analysis of variance and post hoc Scheffé tests]. Bars indicate means ± SEM of five to seven experiments.

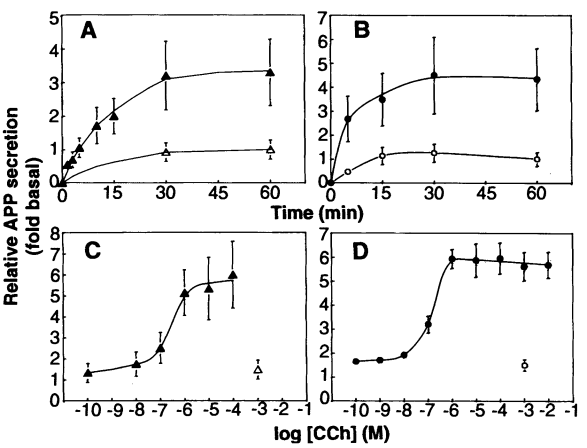

Fig. 2. Time-dependent (**A** and **B**) and concentration-dependent (**C** and **D**) release of APP derivatives by human 293 cell lines transfected with the genes for the human m1 (triangles) and m3 (circles) mAChR subtypes in response to receptor activation by carbachol (CCh). (A and B) Filled symbols, CCh; open symbols, vehicle controls. (C and D) Filled symbols, CCh; open symbols, CCh in the presence of atropine (10 μM). For the time courses, the carbachol concentration was 1 mM; for the dose-response curves, a 30-min incubation period was used. Data are means ± SEM of representative experiments with triplicate cell culture dishes (A to C) and means of two experiments with triplicate culture dishes (D).

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). The effect of carbachol was also inhibited by staurosporine, suggesting that mAChR-coupled APP release may be mediated by protein kinase activation (Fig. 1, A, B, and E). Stimulation of m1 and m3 receptors activates protein kinase C (PKC) by increasing diacylglycerol formation (*14*), which, along with inositol phosphate, 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 NH_2-terminal APP derivatives and the abundance of cell-associated COOH-terminal APP cleavage products in PC-12 cells and to phosphorylate APP at Ser^{655} 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-

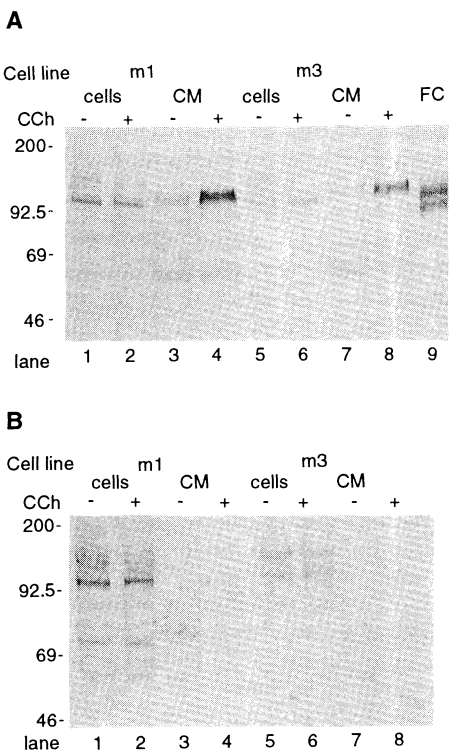

Fig. 3. Western blot of cell-associated (cells) and secreted (CM, conditioned media) APP from m1 mAChR– and m3 mAChR–transfected human 293 cells with the use of (**A**) the monoclonal antibody 22C11 and (**B**) a polyclonal antiserum directed against the COOH-terminus of APP (anti-C_8) (*19*). FC, frontal cortex (20 μg of protein), was obtained at autopsy from an 85-year-old woman who had no evidence of neurologic disease. Molecular size markers are shown at the left (in kilodaltons).

course experiments strongly suggest that preexisting cellular APP was processed in response to receptor activation [using pulse-chase methods, Weidemann *et al.* (*5*) showed that secreted forms of APP do not appear in the medium until 45 min after labeling]. Stimulation of the release of APP derivatives by carbachol showed a clear dose-response relation (Fig. 2, C and D), and the median effective concentration (EC_{50}) values of 0.4 μM for the m1- and 0.15 μM for the m3-transfected cells were virtually identical to those obtained for 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 antiserum 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 antiserum revealed the expected pattern of APP and APP fragments (Fig. 3B), which was similar to that described in human brain cortex (*20*). This antiserum 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 NH_2-terminal APP derivatives. This mechanism presumably is mediated by receptor-coupled activation of PKC. This is the first report describing the effect of neurotransmitter receptor activation on APP processing, which appears to be changed in the brains of individuals with Alzheimer's disease. Our results lead to the hypothesis that abnormal APP processing in Alzheimer's disease could in part be due to altered neurotransmitter receptor control of APP-producing cholinergic target cells expressing m1 and m3 mAChR. Further study of receptor-controlled APP processing in this cell culture model system should provide additional insight into both the regulation of APP processing and the possibility that alterations of this mechanism lead to the generation of amyloidogenic (*8*) or toxic (*21*) APP fragments.

REFERENCES AND NOTES

1. D. J. Selkoe, *Neuron* **6**, 487 (1991).
2. G. G. Glenner and C. W. Wong, *Biochem. Biophys. Res. Commun.* **120**, 885 (1984); C. L. Masters *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4245 (1985); D. J. Selkoe *et al.*, *J. Neurochem.* **46**, 1820 (1986).
3. J. Kang *et al.*, *Nature* **325**, 733 (1987).
4. R. E. Tanzi *et al.*, *ibid.* **331**, 528 (1988); P. Ponte *et al.*, *ibid.*, p. 525; N. Kitaguchi *et al.*, *ibid.*, p. 530; G. König *et al.*, *J. Biol. Chem.* **267**, 10804 (1992).
5. A. Weidemann *et al.*, *Cell* **57**, 115 (1989); M. R. Palmert *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6338 (1989).
6. T. Oltersdorf *et al.*, *Nature* **341**, 144 (1989); F. S. Esch *et al.*, *Science* **248**, 1122 (1990); S. S. Sisodia, E. H. Koo, K. Beyreuther, A. Unterbeck, D. L. Price, *ibid.*, p. 492; J. P. Anderson *et al.*, *Neurosci. Lett.* **128**, 126 (1991); R. Wang *et al.*, *J. Biol. Chem.* **262**, 16960 (1991).
7. C. Haass, A. Y. Hung, D. J. Selkoe, *J. Neurosci.* **11**, 3783 (1991); C. Haass, E. H. Koo, A. Mellon, A. Y. Hung, D. J. Selkoe, *Nature* **356**, 500 (1992).
8. S. Estus *et al.*, *Science* **255**, 726 (1992); T. E. Golde, S. Estus, L. H. Younkin, D. J. Selkoe, S. G. Younkin, *ibid.*, p. 728.
9. E. G. Peralta, A. Ashkenazi, J. W. Winslow, J. Ramachandran, D. J. Capon, *Nature* **334**, 434 (1988). The nomenclature for the mAChR subtypes used in this report follows the recommendation of the Muscarinic Receptor Nomenclature Committee [*Trends Pharmacol. Sci.* (suppl.), p. vii (1989)].
10. T. Oltersdorf *et al.*, *J. Biol. Chem.* **265**, 4492 (1991).
11. Human 293 cell lines were transfected with the human genes for the m1, m2, m3, and m4 mAChR subtypes as well as for neomycin resistance as described (*9*) and grown in Dulbecco's minimum essential medium–F-12 medium containing 10% fetal bovine serum (Gibco) in the presence of the neomycin analog G418 (0.5 mg/ml) (Geneticin, Gibco). Nontransfected human 293 cells were obtained from the American Tissue Type Collection (CRL 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-D-lysine–coated [relative molecular mass >300,000; 0.1 mg per dish] culture dishes were washed with serum-free N-2 medium (Gibco) and incubated for 30 or 60 min in serum-free N-2 with or without 1 mM carbachol (Sigma), 10 μM 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 per dish was measured with the bicinchoninic 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.
12. We compared immunoreactive bands densitometrically with the use of an LKB Ultroscan 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 analyses of variance and post hoc Scheffé tests as indicated.
13. J. Sandmann, E. G. Peralta, R. J. Wurtman, *J. Biol. Chem.* **266**, 6031 (1991).
14. Y. Nishizuka, *Science* **233**, 305 (1986).
15. M. J. Berridge and R. F. Irvine, *Nature* **341**, 197 (1989).

16. G. L. Caporaso, S. E. Gandy, J. D. Buxbaum, T. V. Ramabhadran, P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3055 (1992); J. D. Buxbaum *et al.*, *ibid.* **87**, 6003 (1990); T. Suzuki, A. C. Nairn, S. E. Gandy, P. Greengard, *Neuroscience* **48**, 755 (1992).
17. S. Gandy, A. J. Czernik, P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6218 (1988).
18. R. M. Nitsch *et al.*, unpublished data.
19. Cells were lysed on ice in a buffer containing 2% Triton X-100, 2% SDS, 0.1 M tris (pH 6.8), 15% glycerol, EDTA (5 mM), phenylmethylsulfonyl fluoride (2 mM), aprotinin (10 μM), leupeptin (1 μg/ml), pepstatin (0.1 μg/ml), and tosyl-L-lysine chloromethyl ketone (1 μg/ml) (all from Sigma), ultrasonicated, boiled (5 min), and diluted 1:1 in SDS-free loading buffer. Equal amounts of cell protein (20 μg per lane) and media corresponding to 300 μg of total cell protein were separated on 7.5% SDS-polyacrylamide gels, and Western blots were performed as described in (*5*). For each treatment group represented on the Western blots, proteins secreted into the conditioned media and cell-associated proteins were obtained from the identical culture dishes.
20. D. J. Selkoe *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7341 (1988).
21. N. W. Kowall, M. F. Beal, J. Busciglio, L. K. Duffy, B. A. Yankner, *ibid.* **88**, 7247 (1991).
22. We thank E. G. Peralta for providing cell lines and D. J. Selkoe and K. Naujoks (Boehringer Mannheim) for providing antibodies. We are grateful to C. Bilmazes for technical assistance and to R. E. Tanzi for critically reading the manuscript. Supported by the National Institute of Mental Health, the National Institute on Aging, and the Center for Brain Sciences and Metabolism Charitable Trust. R.M.N. is the Hoffman Fellow in Alzheimer's disease at Massachusetts General Hospital.

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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—Ser^{248}, Leu^{250}, Ser^{252}, and Thr^{254}—in M2, a membrane-spanning segment of the α subunit, are exposed in the closed channel. Thus $\alpha^{248-254}$ 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, Leu^{251} 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 pseudosymmetric (*3*) ring around a central channel (*4*). Each subunit contains four membrane-spanning segments, M1 through M4 (*3*, *5*); both the NH_2-terminus and the COOH-terminus 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 Thr^{244} of the *Torpedo* α subunit altered cation selectivity (*10*, *11*), mutations of residues homologous to Ser^{248} and Ser^{252} of the α subunit influenced the binding of the channel blocker QX-222 (*12*), and mutation of a residue homologous to *Torpedo* α subunit Leu^{251} 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 Ser^{248} (*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 Leu^{251} and a residue in the γ subunit (*17*) homologous to α subunit Thr^{244}. A residue flanking M2, α subunit Glu^{262}, 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 segments 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 the aspartate receptor (*20*), colicin (*21*), and bacteriorhodopsin (*22*).

The small, charged, sulfhydryl-specific reagents, which we synthesized, are the three methanethiosulfonate (MTS) derivatives, $CH_3SO_2SCH_2CH_2NH_3^+$ (MTSEA), $CH_3SO_2SCH_2CH_2NMe_3^+$ (MTSET), and $CH_3SO_2SCH_2CH_2SO_3^-$ (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 $-SCH_2CH_2R$ 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 nonprotein sulfhydryls (*23*).

We mutated one at a time nine consecutive residues in M2 (246 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

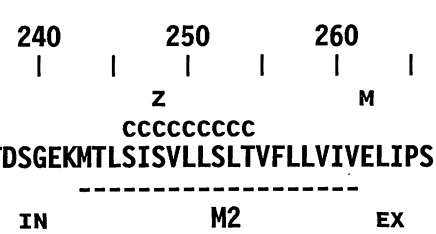

Fig. 1. Partial sequence of the mouse muscle ACh receptor α subunit, numbered by alignment with *Torpedo* α subunit. M2 segment underlined; EX, extracellular; IN, intracellular; Z, labeled by chlorpromazine (*15*) and triphenylmethylphosphonium (*14*) and M, labeled by meproadifen mustard (*19*), all in *Torpedo* α; C, mutated to cysteine as described in text. Single-letter codes for the amino acids are used (*37*).

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