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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.
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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.

23 April 1992; accepted 28 July 1992

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

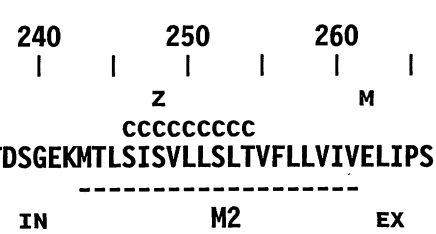

Fig. 1. Partial sequence of the mouse muscle ACh receptor α subunit, numbered by alignment with *Torpedo* α subunit. M2 segment underlined; EX, extracellular; IN, intracellular; Z, labeled by chlorpromazine (*15*) and triphenylmethylphosphonium (*14*) and M, labeled by meproadifen mustard (*19*), all 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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later, all mutants gave robust responses to ACh (*27*). We tested the susceptibility of wild-type and mutant receptors to irreversible inhibition by a 1-min application of the MTS derivatives in the presence and absence of ACh (Fig. 2A). During 1-min applications of ACh, slow desensitization caused a decline from peak current that ranged from 3 to 42%; therefore, a large fraction of the receptors were opening during the entire 1-min period (Fig. 2B).

MTSEA, added for 1 min in the absence of ACh, irreversibly inhibited the mutants S248C, L250C, and S252C significantly more than the wild type (*28*) (Fig. 3A). We infer that the side chains of the corresponding wild-type residues line the channel and are accessible from the extracellular end of the channel in its closed state. The accessibility of Ser^{248} and Ser^{252} is consistent with previous results of labeling and mutagenesis (*12, 14–18*); the accessibility of Leu^{250}, however, is surprising not only because there is no indication from previous work that Leu^{250} is exposed in the channel but also because its accessibility is incompatible with an α-helical conformation for residues from 248 to 252. An α-helical conformation of all four membrane-spanning segments, including M2, has been widely assumed. If this were the case, Leu^{250} would be on the side of an α-helix opposite to Ser^{248} and Ser^{252}. The side chains of all three residues, however, would be on the same side of a segment in a β-strand conformation.

Because T254C was inhibited more by a 1-min application of MTSEA than was the wild type, we tested a 5-min application of MTSEA on T254C, S246C, and wild type. The longer exposure to MTSEA resulted in 23% ± 5% (n = 3) irreversible inhibition of T254C, 9% ± 0% (n = 2) of S246C, and 8% ± 4% (n = 3) of wild type. T254C was inhibited more than wild type (P < 0.02), but S246C was not. We infer that in the channel of the wild-type receptor the side chains of Ser^{248}, Leu^{250}, Ser^{252}, and Thr^{254} are exposed and probably are in a β-strand conformation.

Because the cationic MTSEA can penetrate into the closed channel from its extracellular end at least to Ser^{248}, the gating element that blocks cation conduction in the closed state of the channel must be closer to the cytoplasmic end of the channel than Ser^{248}.

In the presence of ACh as in its absence, MTSEA irreversibly inhibited S248C, L250C, and S252C (Fig. 3B). In contrast, L251C was strongly irreversibly inhibited by MTSEA when applied in the presence of ACh (Figs. 2A and 3B), but not in its absence (Fig. 3A). The ACh-dependent accessibility of the cysteine residue substituted for Leu^{251} reflects a structural change in the channel lining attendant on channel opening or desensitization or both. The gate itself, however, is closer to the cytoplasmic end of the channel than Leu^{251}.

In the presence of ACh, three consecutive residues—Leu^{250}, Leu^{251}, and Ser^{252}—are accessible to MTSEA. Three consecutive residues are simultaneously accessible to solvent when they are located at the ends of β strands or α helices or in turns, bends,

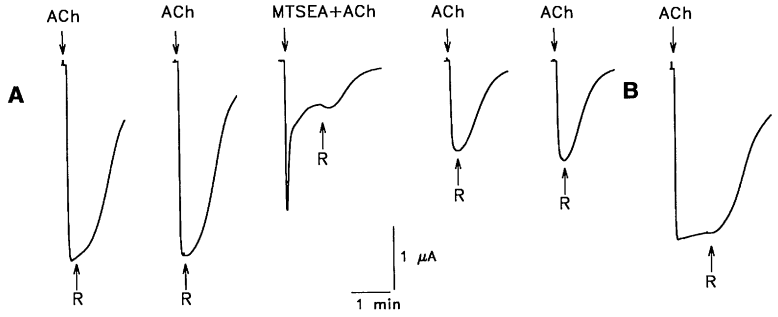

Fig. 2. The effect of MTSEA plus ACh on the response of mutant L251C. (**A**) The susceptibility of wild type and all mutants to the MTS derivatives was tested by recording the voltage-clamp current while perfusing with the following sequence of solutions: 20 μM ACh (10 s), Ringer solution (R) (5 min), 20 μM ACh (10 s), Ringer solution (5 min), MTS derivative ± 20 μM ACh (1 min), Ringer solution (5 min), 20 μM ACh (10 s), Ringer solution (5 min), 20 μM ACh (10 s). The responses of a single oocyte are shown: Two precede and two follow a 1-min application of 2.5 mM MTSEA plus 20 μM ACh. Solution changes are at the arrows. (**B**) For comparison, the response of the same mutant as in (A) in another oocyte to a 1-min application of 20 μM ACh and no MTSEA.

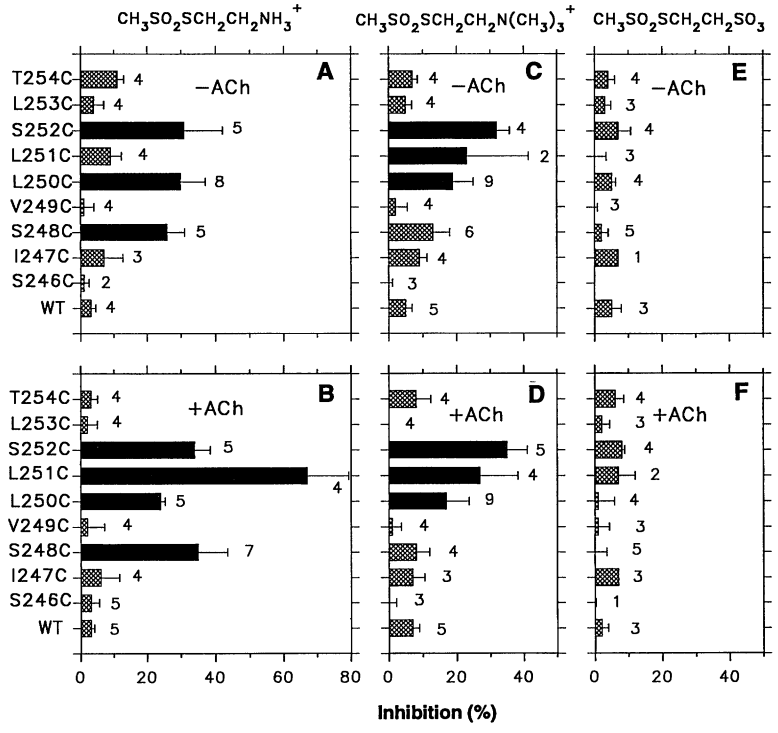

Fig. 3. The irreversible inhibition of the response of wild-type and mutant receptors to ACh resulting from a 1-min application of MTS derivatives. The derivatives added (2.5 mM MTSEA, 1 mM MTSET, and 10 mM MTSES) are indicated along the tops of the panels, and the presence or absence of ACh during the addition of the MTS derivative is indicated within each panel. The fractional inhibition was calculated as 1 − (mean final response/mean initial response). This inhibition was corrected by subtracting the decline in the average response for the same receptor when 20 μM ACh alone (no MTS derivative) was applied to the oocyte for 1 min. These corrections ranged from 0 to 6%, except for S246C for which the correction was 9%. The means, SEMs, and number of independent experiments are shown. Three-way analysis of variance showed significant three-way interactions (P < 0.001). One-way analysis of variance showed significant (P < 0.001) differences between mutants within each of six groups of the three MTS derivatives with and without ACh. The differences between the means were evaluated by the least significant difference procedure. Solid bars, mutants for which the inhibition was significantly different (P < 0.05) than for wild type.

and loops (29). Alternatively, Leu^{250}, Leu^{251}, and Ser^{252} are not necessarily simultaneously exposed in a single α subunit because in the presence of ACh we are sampling receptors in any of three states: open, closed, and desensitized. In either case, flexibility is required in the channel structure around Leu^{251}.

MTSET contains a quaternary ammonium group and is an agonist. In the wild type and in L251C, 1 mM MTSET induced approximately 75% of the current induced by 20 μM ACh. Consistent with its agonist activity, MTSET inhibited similarly in the presence and absence of ACh (Fig. 3, C and D). MTSET irreversibly inhibited S252C, L251C, and L250C, as did MTSEA in the presence of ACh. Unlike MTSEA, MTSET did not inhibit S248C. This difference could be due to the difference in the size of the head groups. The $-N(CH_3)_3$ group of MTSET has a diameter of about 0.58 nm compared to 0.36 nm for the $-NH_3$ group of MTSEA, and the channel may narrow near its cytoplasmic end (10, 30).

The anionic MTSES had no significant effects on wild-type or mutant channels either in the presence or absence of ACh (Fig. 3, E and F). The $-SO_3$ group, although smaller than the $-N(CH_3)_3$ group, is larger than the $-NH_3$ group. If size alone were the criterion, then MTSES would have access to at least the same residues as MTSET. The lack of effect of MTSES on S252C, L251C, and L250C, therefore, is due to selection against anions either in this region or between the extracellular end of the channel and this region.

From Ser^{248} to Thr^{254}, which we have inferred form a β strand, the effect of cysteine substitution on the K_{app} for ACh alternates (Fig. 4, A and B). K_{app} was 2.6 ± 0.4 μM (n = 12) for wild type and was 0.2 to 129 μM for the mutants. The K_{app}'s of the mutants in which the residue altered faces the closed channel—S248C, L250C, S252C, and T254C—were greater than that of the wild-type receptor, whereas the K_{app}'s of S246C, L251C, and L253C were less. The effect depends more on the position of the residue altered than on the identity of the side chain in the wild-type receptor.

Because these residues are far from the ACh binding sites, the effects on K_{app} probably result from changes in the rates of channel opening and closing rather than in ACh binding affinity. Thus we can estimate the effect of mutation on the free-energy difference between the open and closed states (Fig. 4B, upper scale) (31). Mutation of the residues that face the channel lumen in the closed state increases the free-energy cost of channel opening; mutation of residues that face the protein interior either decreases the free-energy cost of channel opening, in the cases of Leu^{253} and Leu^{251}, or has no effect on the free-energy change, in the case of Val^{249}. In particular, the hydrophobic interactions of Leu^{251} and Leu^{253} with the interior of the protein in the closed state and their less favorable interactions in the open state may restrain spontaneous opening of the channel. The binding of ACh provides the energy to overcome this energy barrier. The substitution of the smaller cysteine at these positions may lower this barrier.

The only channel for which a high-resolution structure has been elucidated is a β-strand structure. The porin channel is formed by a 16-strand β barrel (32). In addition, the pore of the voltage-gated K^+ channel may be formed by an eight-strand β barrel (33). If Ser^{248} to Thr^{254} of the ACh receptor α subunit forms a β strand and there is fivefold pseudosymmetry around the channel, then the analogous residues in the β, γ, and δ subunits also would form β strands. A symmetrical, five-strand, parallel β cylinder is formed by seven residues of human rhinovirus-14 capsid VP3 subunits (34), and, although this structure has a lumen less than 0.6 nm in diameter, a similar structure with greater tilt of the strands would have a larger lumen. Another possible fivefold symmetrical structure would be a ten-strand, antiparallel β barrel (35), which, in the case of the ACh receptor, would require a contribution by a membrane-spanning segment in addition to M2, for instance, M1 (36). Our approach will permit us to determine whether the residues of M1 face the channel lumen. Moreover, the approach should be applicable to all cloned ion channels.

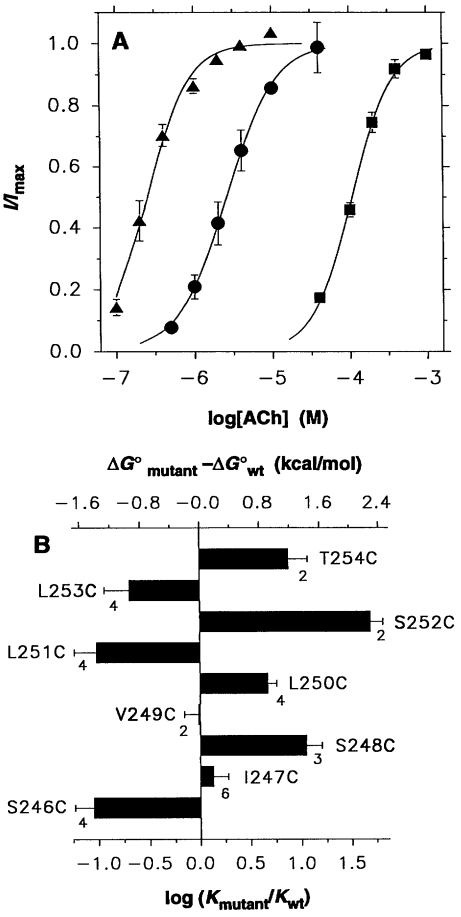

Fig. 4. (**A**) Representative normalized dose-response curves for wild type (circles), L251C (triangles), and S252C (squares). Each curve is from a single oocyte. Each concentration of ACh was applied twice and the average peak currents plotted. The solid lines were calculated by fitting the peak currents to the Hill equation, $I = I_{max}/\{1 + (K_{app}/[ACh])^n\}$, with the Marquardt-Levenberg algorithm. I_{max} was −2.92 μA for wild type, −3.43 μA for L251C, and −1.56 μA for S252C. (**B**) The mean K_{app} for the mutants relative to the mean K_{app} for the wild-type receptor. The standard error in the ratio and the number of independent determinations of K_{app} for each mutant are shown. $K_{app,wt}$ = 2.6 ± 0.4 μM (n = 12). The values on the upper scale are $2.303*RT*\log(K_{app,mutant}/K_{app,wt})$; these values are the differences in the standard Gibbs free energies between the open and closed states of mutant receptors, $\Delta G°_{mutant}$, minus the difference in standard Gibbs free energies between the open and closed states of the wild-type receptor, $\Delta G°_{wt}$ (31).

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26. Polymerase chain reaction mutagenesis was performed in a cassette defined by the restriction enzymes Dra III and Ppu MI [R. M. Nelson and G. L. Long, *Anal. Biochem.* **180**, 147 (1989); O. P. Kuipers, H. J. Boot, W. M. de Vos, *Nucleic Acids Res.* **19**, 4558 (1991)]. The mutant cassettes were sequenced in both directions. Capped mRNA for each subunit was transcribed with SP6 polymerase under standard conditions (Promega) from cDNAs in pSP64T plasmid. We prepared *Xenopus laevis* oocytes by incubating small pieces of ovary in collagenase (20 mg/ml) (Sigma Type I) in OR3 media [1:2 dilution of Lebovitz L-15 media, 1 mM glutamine, gentamycin (100 μg/ml), 15 mM Hepes, pH 7.6 with NaOH] for 20 min at 17°C (P. Brehm, personal communication). The ovaries were washed five times in OR3, and the oocytes were dissected. One day later, they were injected with 50 nl of mRNA (200 pg/nl) mixed in a ratio of 2:1:1:1 (α:β:γ:δ).
27. ACh-induced currents were recorded with a two-electrode voltage clamp at a holding potential of −40 mV. Electrodes were filled with 3 M KCl and had a resistance of less than 2 megaohms. The oocytes were perfused at 5 ml/min with Ca^{2+}-free Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 1 μM atropine, 10 mM Hepes, pH 7.5 with NaOH), in which all reagents were applied.
28. In about 50% of the experiments, the second response after the application of the MTS derivative was greater than the first. We recorded the responses of L251C and of S248C for about 1 hour after the application of MTSEA. In oocytes in which we observed recovery from the inhibition, the half-time was about 40 min. This recovery was not due to reductive or hydrolytic cleavage of the disulfide bond because we observed the same rate of recovery from inhibition due to the reactions of 2 mM dithiothreitol followed by 1 μM 4-(*N*-maleimido)benzyltrimethylammonium (MBTA), in which the MBTA forms a stable thioether with Cys^{192} or Cys^{193} at the ACh binding site [P. N. Kao *et al.*, *J. Biol. Chem.* **259**, 11662 (1984)]. We conclude that the receptor at the oocyte surface is turning over with a half-time of about 40 min.
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31. A simple scheme for receptor activation, ignoring cooperativity, is $A + R_0 = R_1 = R_1^*$, where A is agonist, R_x is receptor with x sites occupied, and * indicates open state. At equilibrium, $[A][R_0]/[R_1] = K$, $[R_1]/[R_1^*] = L$, and $[R_1]^* = [R_{total}]/(1 + L + KL/[A])$. $K_{app} = KL/(1 + L)$, and since $L < 0.1$ [D. Colquhoun and B. Sakmann, *Nature* **294**, 464 (1981)], $K_{app} \cong KL$. Assuming that mutations in M2 affect only the isomerization constant L and not the intrinsic binding constant K, $K_{app,mutant}/K_{app,wt} = L_{mutant}/L_{wt}$. Furthermore, $G^\circ_{open} - G^\circ_{closed} = -RT\ln([R_1^*]/[R_1]) = RT\ln(L)$, where G° is standard Gibbs free energy and wt is wild type.
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37. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
38. We thank H. Andrews, P. Brehm, T. Claudio, C. Czajkowski, S. Goff, W. Hendrickson, J. Hubbard, J. Javitch, H. Lester, I. Lowy, J. Messing, J. Murphy, J. Pittman, M. Reitman, L. Role, B. Sakmann, and S. Siegelbaum for invaluable advice and materials, E. Silva-Herzog for early experiments, and G. Salazar-Jimenez and C. Kaufmann for technical assistance. Supported in part by research grants from the NIH (NS07065) and the Muscular Dystrophy Association. Supported by NIH training grant NS07258 and an MDA Research Fellowship (D.A.S.).

29 July 1992; accepted 16 September 1992

Calcium Channels Coupled to Glutamate Release Identified by ω-Aga-IVA

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Presynaptic calcium channels are crucial elements of neuronal excitation-secretion coupling. In mammalian brain, they have been difficult to characterize because most presynaptic terminals are too small to probe with electrodes, and available pharmacological tools such as dihydropyridines and ω-conotoxin are largely ineffective. Subsecond measurements of synaptosomal glutamate release have now been used to assess presynaptic calcium channel activity in order to study the action of peptide toxins from the venom of the funnel web spider *Agelenopsis aperta*, which is known to inhibit dihydropyridine and ω-conotoxin–resistant neuronal calcium currents. A presynaptic calcium channel important in glutamate release is shown to be ω-Aga-IVA sensitive and ω-conotoxin resistant.

The key step in excitation-secretion coupling in presynaptic nerve terminals is the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels (*1*). Analysis of Ca^{2+} currents in neuronal soma (*2–5*) and in certain nerve terminal preparations (*6–8*) has provided evidence for multiple types of Ca^{2+} channels. The sensitivity of presynaptic Ca^{2+} channels at amphibian (*9*), reptilian (*8*), and avian synapses (*10, 11*) to ω-conotoxin (ω-CgTx) has led to widespread acceptance of a predominant role for N-type channels in neuronal excitation-secretion coupling. However, in mammalian systems, Ca^{2+} entry, neurosecretion, and synaptic transmission are only partially inhibited (*12–15*) or largely resistant to ω-CgTx and dihydropyridines (*16–18*), suggesting that the exocytotic Ca^{2+} channel at most mammalian brain synapses is a distinct subtype.

Venom of the funnel web spider *Agelenopsis aperta* contains toxins that have been shown to inhibit Ca^{2+} channels in vertebrates. These toxins include funnel toxin (FTX) (*19, 20*), a low molecular weight polyamine, and the peptides ω-Aga-IIIA and ω-Aga-IVA, two members of a family of at least four ω-agatoxins (*21*). In mammalian neuronal somata, high-voltage–activated Ca^{2+} channels of the L- and N-types (*22*), as well as those of the P-type (*23*), are blocked by ω-Aga-IIIA. In contrast, ω-Aga-IVA has been reported to be more selective, targeting the P-type currents of cerebellar Purkinje cells and rat sensory neurons (*18*). The potent block of $^{45}Ca^{2+}$ uptake into rat brain synaptosomes by ω-Aga-IVA (*18*) suggests that presynaptic P-type channels may trigger neurosecretion in mammalian brain. We report that ω-Aga-IIIA and ω-Aga-IVA partially (but potently) block [^{3}H]glutamate release from rat brain synaptosomes. These results provide additional pharmacological criteria that can be used to identify presynaptic Ca^{2+} channels involved in excitation-secretion coupling on the basis of sensitivity to ω-Aga-IVA and resistance to ω-CgTx.

The effect of the peptide toxins on presynaptic Ca^{2+} channels was assayed on the basis of the ability to block [^{3}H]glutamate release from synaptosomes (a preparation enriched in intact presynaptic nerve terminals), obtained by homogenizing rat frontal cortex in isotonic sucrose. The terminals were incubated in a solution containing [^{3}H]glutamate to metabolically label an exocytotic pool that can be released in a Ca^{2+}-dependent manner when depolarized by an increase in the external K^+ concentration. The loaded synaptosomes were immobilized on a glass fiber filter and secured in a chamber where they were superfused with solutions of defined composition. The effluent stream containing released radioactivity was collected in 70-ms segments in vials juxtaposed on the perimeter of a phonograph turntable. The combination of superfusion flow rates of 1 to 2

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