

mutation in the nuclear genome. The mutants were classified into six complementation groups, *wav1* to *wav6* (Table 1). The *wav1-1* mutant showed neither wavy growth on the angled agar surface (Fig. 3D) nor root tip rotation (Fig. 3F). In contrast, the *wav2-1* mutant developed waves of shorter pitch than those of the wild type (Fig. 3B), possibly due to a high rate of root tip rotation (Fig. 3C). The *wav3-1* mutant is non-allelic with *wav2-1* but shows similar short pitch waves (data not shown). The waving pattern of the *wav4-1* mutant is rectangular (Fig. 3G), seemingly due to irregular timing of reversion of root tip rotation (Fig. 3E). The root growth pattern of the *wav5-33* mutant is different from that of the other mutants: the root tips undergo continuous left-handed rotation, so that the roots form clockwise circles on the agar surface (Fig. 3, H and I). The *wav6-52* mutant is similar to the *wav1-1* mutant, lacking wavy growth on the angled surface (data not shown). Rotation of the root tips in the *wav2*, *wav3*, *wav4*, and *wav5* mutants could be terminated if the agar plates were shifted back to the vertical position (the *wav5-33* mutant is shown in Fig. 3, H and I, arrow 2). Root gravitropism was found to be normal in *wav1* to *wav4* but absent in *wav5* and greatly reduced in *wav6*. The *wav5* and *wav6* mutant genes are allelic with known root gravitropism genes, *aux1* (3) and *agr1* (4), respectively (Table 1).

Although the mechanism of the reversion of rotation observed in the wild-type plant is not understood, we suggest that reversion of the twisting direction is dependent on correct gravity sensing and responsiveness, and that the mutation in *wav5-33* destroys the ability to undergo such reversion. Our results suggest the existence of a signal transduction mechanism, beginning with perception of the touching stimulus at the root tip followed by signal transfer from the root tip to the elongation zone and the periodic reversion of rotation that results in wavy growth. In this model, the genetic defect in *wav1* could be anywhere downstream of perception of the obstacle. The lesions in *wav2*, *wav3*, and *wav4* may be in signal processing or in the twisting response of the root tip.

#### REFERENCES AND NOTES

1. G. P. Redei, *Annu. Rev. Genet.* **9**, 111 (1975); E. M. Meyerowitz, *ibid.* **21**, 93 (1987); *Cell* **56**, 263 (1989); C. Somerville, *Plant Cell* **1**, 1131 (1989).
2. *Arabidopsis thaliana* Landsberg wild-type seeds were mutagenized by ethyl methanesulfonate (EMS). About 3,000 seeds were soaked in EMS solution (0.3% v/v in water) for 16 to 24 hours at room temperature, washed repeatedly with water, and sown in pots. A portion of the seeds (about 10,000 M2 seeds) harvested from the mutagenized plants were sown on agar plates for mutant screening. Eleven strains with apparently abnormal wavy

growth were isolated, and these were divided into six complementation groups, *wav1* to *wav6* (*wav* stands for wavy growth). Basic genetic analysis was performed according to the procedures described by M. K. Komaki *et al.* [*Development* **104**, 195 (1988)].

3. E. P. Maher and S. J. B. Martindale, *Biochem. Genet.* **18**, 1041 (1980).
4. C. J. Bell and E. P. Maher, *Mol. Gen. Genet.* **220**, 289 (1990).
5. C. R. Somerville and W. L. Ogren, in *Chloroplast Molecular Biology*, M. Edelman, R. Hallick, N.-H.

Chua, Eds. (Elsevier, New York, 1982), pp. 129–138.

6. We thank C. J. Bell for providing seeds of the *aux1* and *agr1* mutants and for discussion and critical reading of the manuscript. This work was supported, in part, by grants from the Japanese Ministry of Education, Science, and Culture and by funds from the Naito Foundation for the Promotion of Science and from the Ciba-Geigy Foundation for the Promotion of Science.

9 April 1990; accepted 5 July 1990

## Mutations Affecting TEA Blockade and Ion Permeation in Voltage-Activated K<sup>+</sup> Channels

RODERICK MACKINNON\* AND GARY YELLEN

**Voltage-dependent ion channels are responsible for electrical signaling in neurons and other cells. The main classes of voltage-dependent channels (sodium-, calcium-, and potassium-selective channels) have closely related molecular structures. For one member of this superfamily, the transiently voltage-activated Shaker H4 potassium channel, specific amino acid residues have now been identified that affect channel blockade by the small ion tetraethylammonium, as well as the conduction of ions through the pore. Furthermore, variation at one of these amino acid positions among naturally occurring potassium channels may account for most of their differences in sensitivity to tetraethylammonium.**

**T**HE VOLTAGE-ACTIVATED K<sup>+</sup> channels are a diverse family of channel proteins that share the common feature of a K<sup>+</sup>-selective pore. In searching for the specific amino acid residues that line the pore of these K<sup>+</sup> channels, we have been guided by work (1, 2) on the interaction of the Shaker H4 K<sup>+</sup> channel protein with charybdotoxin (CTX), a protein component of scorpion venom (3). Many mutations in the linker between the S5 and S6 regions of the protein (Fig. 1) affect CTX binding significantly (1, 2). Some of the effects of mutations on CTX binding can be explained by a simple, through-space electrostatic mechanism, whereas others probably affect the intimate protein-protein contact between channel and toxin (2). Because CTX occludes the pore (4) but is physically large (5), we presume that the sites it has identified lie in the outer vestibule of the pore entryway. In this study, we used tetraethylammonium (TEA), a small open channel blocker, to search for residues that may line the narrower region of the pore.

Ammonium ion can pass through most K<sup>+</sup> channels, whereas its quaternary derivative TEA cannot, resulting in the blockade

of most voltage-activated K<sup>+</sup> channels (6, 7). The effect of intracellularly applied TEA is relatively uniform among different voltage-activated K<sup>+</sup> channels. By contrast, the efficacy of extracellular TEA in blocking K<sup>+</sup> channels in different preparations is quite variable: for example, K<sup>+</sup> channels at frog node of Ranvier are blocked by millimolar concentrations of TEA, whereas squid axon K<sup>+</sup> channels are unaffected even by much higher concentrations (8).

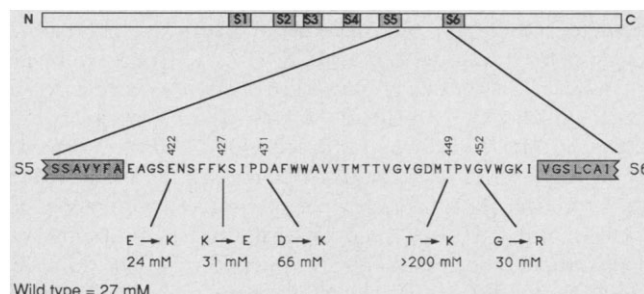
We examined the effect of mutations in the S5–S6 linker on channel blockade by externally applied TEA (Fig. 1). The wild-type Shaker H4 channel has low sensitivity to TEA (27 mM ± 5; mean ± SD, *n* = 3). Most of the initial mutations we made had no effect on TEA blockade (Fig. 1); the two exceptions were the substitution of a lysine at either of positions 431 and 449. At position 431, a lysine substitution (mutant D431K) reduced TEA sensitivity by about a factor of 2 (to 66 mM ± 10, *n* = 3). The effect of replacing the amino acid at position 449 (T449K) with lysine was even more dramatic, effectively abolishing the inhibition by TEA (Fig. 2).

To investigate further the importance of these sites for TEA blockade, we prepared mutants with other amino acid substitutions at the 431 and 449 positions (Table 1). At position 431, a single-charge change (D431N) had a weaker effect on TEA sensitivity than the double-charge change (D431K), and a conservative mutation that

R. MacKinnon, Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115.  
G. Yellen, Howard Hughes Medical Institute and the Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

\*To whom correspondence should be addressed.

**Fig. 1.** Amino acid sequence of the S5–S6 linker of the Shaker H4 channel, and the effects of mutations on inhibition by external TEA. S1 to S6 are hydrophobic regions of the Shaker protein thought to span the membrane (19, 20); the functional protein is probably an oligomer of the polypeptide shown here. A region between S5 and S6 was called



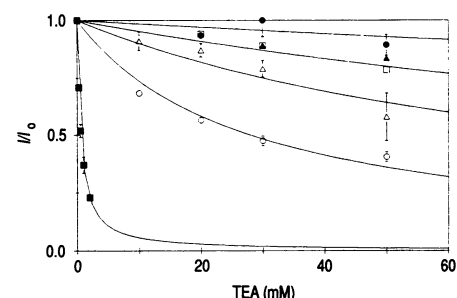
H5 and considered a possible transmembrane sequence by Tempel *et al.* (19). The locations of mutants studied in this region are indicated, as are their  $EC_{50}$  values for inhibition by TEA. Oocytes injected with RNA for each mutation (21) were voltage-clamped with a two-microelectrode amplifier (Axoclamp; Axon Instruments, Foster City, California). Shaker  $K^+$  channel currents were recorded with a p/4 leak subtraction (22) and their amplitudes at a fixed voltage and time (0 to +20 mV; 5 to 10 ms) were measured in the absence and presence of TEA at concentrations from 0 to 50 mM. We calculated the  $EC_{50}$  from at least four determinations on each of at least two oocytes (SD and  $n$  are in Table 1); values did not vary significantly with voltage, nor did they depend on the exact time of the analysis. The recording saline contained 96 mM NaCl, 2 mM KCl, 0.3 mM  $CaCl_2$ , 1 mM  $MgCl_2$ , and 5 mM Hepes (pH 7.6); TEA chloride was substituted for an equivalent amount of NaCl.

did not alter charge (D431E) did not affect the TEA sensitivity. This pattern is consistent with an electrostatic effect of these mutations on TEA block.

The TEA sensitivity of these  $K^+$  channels depends much more critically on the specific amino acid at the 449 position (Fig. 2): substitution of arginine or valine for the threonine reduced binding dramatically (although not as much as substitution with lysine); substitution with glutamine produced a modest reduction (about threefold) in sensitivity; and substitution with tyrosine increased the sensitivity of the channel to TEA by about 50-fold. Although both of

the positively charged substitutions (lysine and arginine) reduced TEA sensitivity substantially, substitutions at this position do not affect TEA block primarily by an electrostatic mechanism, because mutations that produced no change in charge could also alter the sensitivity of the channel.

We next investigated the effects of mutations in the S5–S6 linker on the single-channel conductance of the  $K^+$  channels. We studied single-channel currents through wild-type and mutant Shaker channels with the excised patch recording method (9). To observe outward and inward passage of  $K^+$  ions through the pore, we recorded single-



**Fig. 2.** Effect of mutations at amino acid position 449 of the Shaker H4 channel on TEA block.  $K^+$  current is plotted in the presence of the indicated TEA concentration as a fraction of the current in the absence of TEA ( $I_0$ ). Methods were as described in Fig. 1. ●, Lys; ▲, Val; □, Arg; △, Gln; ○, wild-type Thr; and ■, Tyr.

channel currents with 100 mM  $K^+$  on both sides of the membrane (Fig. 3). Outward channel openings were observed soon after a depolarizing voltage-clamp step, in a fashion characteristic of the transient Shaker channels (10). We also observed channel openings for a brief period after repolarization to negative voltages; these appear as inward (negative) currents.

In symmetrical  $K^+$  solutions, the wild-type Shaker H4 channel carries more current in the inward direction than in the outward direction, as shown by plotting the single-channel current as a function of voltage. Rectification does not appear to depend on blockade by  $Mg^{2+}$  ions (as it does for some other  $K^+$  channels) (11), since the rectification persists in the complete absence of  $Mg^{2+}$  (12).

Of the point mutations we studied in the S5–S6 linker of the Shaker protein, only mutations at the 449 position appeared to affect single-channel conductance (Fig. 4) (13). Substitution of lysine or arginine at the 449 position dramatically affected the single-channel currents (Figs. 3 and 4B), reducing their magnitude and nearly abolishing the inward rectification (the mutants have a nearly linear current-voltage relationship). This effect on single-channel conductance is what one might expect for a mutation that reduces the net negative surface charge on the outside of the membrane (14)—a substantial reduction in inward current accompanied by a smaller reduction in outward current. The valine and tyrosine mutants have wild-type single-channel conductance; however, the glutamine mutant has a reduced conductance, like that of the arginine and lysine mutants. This last result argues against a simple electrostatic mechanism for the effect of mutations at 449 on single-channel conductance.

The comparative study of Stühmer and colleagues (15) on a family of homologous  $K^+$  channels from rat brain showed that one

**Table 1.** Effect of mutations in the S5–S6 linker on TEA block and conductance. Values are reported as mean  $\pm$  SD with number of determinations in parentheses. Single-channel currents ( $i$ ) are reported for  $\pm 80$  mV holding potential. The far right column identifies other cloned  $K^+$  channels with the same residue at the 449 position; the  $EC_{50}$  for TEA block is in parentheses. The  $EC_{50}$  values for the RCK clones are from (15); the *drk1* value is from (16). ND, not done. Amino acids are abbreviated as follows: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

Position and mutation	$EC_{50}$ (TEA) (mM)	$i(-80 \text{ mV})$ (pA)	$i(+80 \text{ mV})$ (pA)	Equivalent natural $K^+$ channels (TEA $EC_{50}$ in mM)
Wild type	$27 \pm 5$ (3)	$-1.98 \pm 0.24$ (5)	$1.18 \pm 0.16$ (3)	
422				
E $\rightarrow$ K	$24 \pm 1$ (2)	$-1.94 \pm 0.14$ (7)	$1.05 \pm 0.04$ (6)	
427				
K $\rightarrow$ E	$31 \pm 4$ (2)	$-2.37 \pm 0.15$ (6)	$1.19 \pm 0.07$ (4)	
431				
D $\rightarrow$ E	$32 \pm 5$ (2)	ND	ND	
D $\rightarrow$ N	$44 \pm 4$ (4)	$-2.33 \pm 0.08$ (12)	$1.13 \pm 0.1$ (7)	
D $\rightarrow$ K	$66 \pm 10$ (3)	ND	ND	
449				
T $\rightarrow$ K	$>200$ (4)	$-1.07 \pm 0.05$ (4)	$0.83 \pm 0.08$ (4)	RCK4 ( $>100$ )
T $\rightarrow$ V	$>150$ (2)	$-1.99 \pm 0.11$ (7)	$0.98 \pm 0.07$ (6)	RCK5 (129)
T $\rightarrow$ Y	$0.59 \pm 0.03$ (3)	$-1.89 \pm 0.12$ (14)	$1.10 \pm 0.06$ (5)	RCK1 (0.6), <i>drk1</i> (10)
T $\rightarrow$ Q	$105 \pm 25$ (5)	$-0.92 \pm 0.1$ (13)	$0.70 \pm 0.09$ (2)	
T $\rightarrow$ R	$>150$ (2)	$-0.81 \pm 0.03$ (6)	$0.95 \pm 0.03$ (2)	
452				
G $\rightarrow$ R	$30 \pm 4$ (2)	$-2.23 \pm 0.32$ (2)	$1.15 \pm 0.01$ (2)	

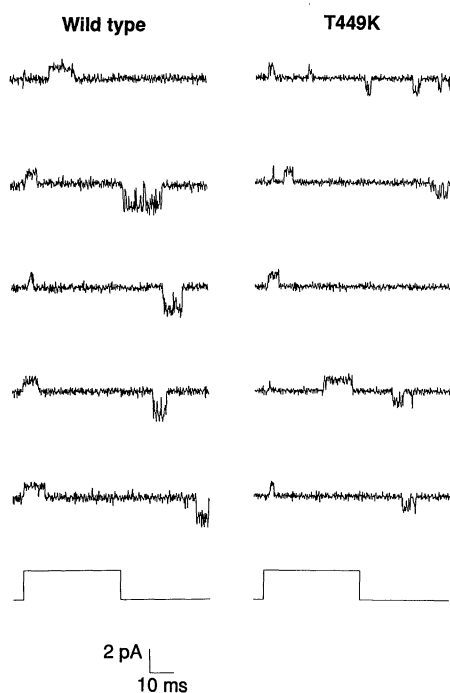
of their four cloned channels (RCK4) has a smaller single-channel conductance than the other three. Although the sequences of the RCK K<sup>+</sup> channels contain many amino acid differences (the pairwise amino acid identity ranges from 59 to 84%), there are few differences in the well-conserved S5–S6 linker. One of these differences is found at the 449 position, where RCK4 has a lysine. Our results suggest that the amino acid variability at this one position may be sufficient to account for the difference in the single-channel conductance of RCK4.

Stühmer and colleagues also noted a difference in the TEA sensitivity of their K<sup>+</sup> channel clones. When we substituted Thr<sup>449</sup> of Shaker H4 with the amino acid found at the corresponding position of RCK1, RCK4, or RCK5, the mutation had the approximate sensitivity of the corresponding RCK clone (Table 1). The TEA sensitivity [median effective concentration (EC<sub>50</sub>) = 0.6 mM] of those K<sup>+</sup> channel proteins with tyrosine at this position (RCK1 and Shaker H4 T449Y) was very high. Another cloned K<sup>+</sup> channel with a tyrosine at this position, *drk1*, also has a relatively high sensitivity to TEA (10 mM) (16); we suggest that the difference between *drk1* channels and the homologs with even higher sensitivity can be accounted for by amino acid differences elsewhere.

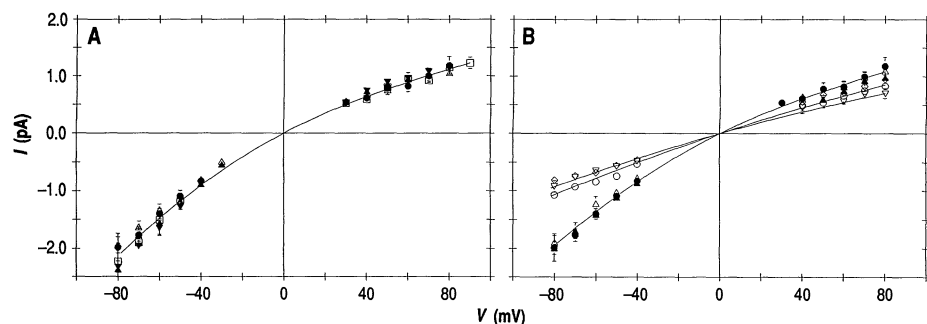
We have identified two positions in the primary structure of the Shaker H4 K<sup>+</sup> channel that appear to be located in or near the outer mouth of the ion conduction pore. Mutations at position 431 affect the ability of CTX (2) and TEA to block channel activity, and the effects on TEA are compatible with an electrostatic mechanism. Mutations at position 449 affect block by CTX (2), block by TEA, and ion conduction. These effects are probably produced not by a simple electrostatic mechanism but rather by a specific alteration in the region of the conduction pathway that binds TEA. Of course, without knowing the three-dimensional structure of both wild-type and mutant channels, we cannot rule out the possibility that these mutations affect the conduction pathway by altering its structure from a remote site. Nevertheless, together with the knowledge that several residues in the vicinities of 431 and 449 have an electrostatic effect on toxin binding, our results suggest that the residues that constitute the pore of the K<sup>+</sup> channel are quite near to these positions.

This hypothesis is consistent with a model proposed by Guy (17), on the basis of a set of structure prediction principles, for the folding of K<sup>+</sup> channels. In the model, the pore is formed by the well-conserved region between 431 and 449, which crosses the membrane twice by making a hairpin turn. This arrangement would place the residues at positions 431 and 449 very near to the external entryway of the conduction pore.

The other members of the voltage-activated ion channel superfamily, Na<sup>+</sup> and Ca<sup>2+</sup> channels, have an amino acid sequence that resembles four repeats of a K<sup>+</sup> channel-like structure. A point mutation located in an analogous region (in the S5–S6 linker) in one repeat of the Na<sup>+</sup> channel protein abolishes inhibition by tetrodotoxin (18). This result is consistent with a similar structure for other voltage-activated ion channels, as one expects from the high degree of sequence homology among the members of this superfamily.



**Fig. 3.** Single-channel currents through Shaker wild-type and T449K channels in symmetrical K<sup>+</sup> solutions. Voltage-clamp currents were recorded for pulses from -80 to +70 mV from excised inside-out patches (9), with a pipette (external) solution containing 96 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.3 mM CaCl<sub>2</sub>, 10 mM 2-(N-morpholino)propane sulfonic acid (MOPS)-KOH (pH 7.1) and a bath solution containing 96 mM KCl, 0.5 mM MgCl<sub>2</sub>, 1 mM EGTA, and 10 mM MOPS-KOH (pH 7.1). Capacitive and leakage currents were eliminated by digital subtraction of averaged blank records.



**Fig. 4.** Single-channel current (*I*)-voltage (*V*) relations for Shaker wild-type and mutant channels. (**A**) Currents for (●) wild-type, (Δ) E422K, (▲) K427E, (▼) D431N, and (□) G452R channels. (**B**) Currents for (●) wild-type, (Δ) T449Y, (▲) T449V, (○) T449K, (▼) T449Q, and (◇) T449R channels. All currents were recorded as in Fig. 3 and the amplitudes were determined by inspection. All determinations were made by an investigator ignorant of the mutation and the voltage being studied.

#### REFERENCES AND NOTES

1. R. MacKinnon and C. Miller, *Science* **245**, 1382 (1989).
2. R. MacKinnon *et al.*, *Neuron*, in press.
3. C. Miller *et al.*, *Nature* **313**, 316 (1985).
4. R. MacKinnon and C. Miller, *J. Gen. Physiol.* **91**, 335 (1988); C. Miller, *Neuron* **1**, 1003 (1988).
5. The dimensions of CTX are approximately 25 by 15 Å [W. Maszefski, Jr., A. G. Redfield, D. R. Hare, C. Miller, *Science* **249**, 521 (1990)].
6. C. M. Armstrong and L. Binstock, *J. Gen. Physiol.* **48**, 859 (1965); B. Hille, *ibid.* **50**, 1287 (1967); P. R. Stanfield, *Rev. Physiol. Biochem. Pharmacol.* **97**, 1 (1983).
7. B. Hille, *Ionic Channels in Excitable Membranes* (Sinauer, Sunderland, MA, 1984).
8. C. M. Armstrong and B. Hille, *J. Gen. Physiol.* **59**, 388 (1972).
9. O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pflügers Arch.* **391**, 85 (1981).
10. C. K. Solc, W. N. Zagotta, R. W. Aldrich, *Science* **236**, 1094 (1987).
11. C. A. Vandenberg, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2560 (1987); M. Horie, H. Irisawa, A. Noma, *J. Physiol. (London)* **387**, 251 (1987); S. Ciani and B. Ribalet, *J. Membr. Biol.* **103**, 171 (1988).
12. R. MacKinnon and G. Yellen, unpublished data.
13. The D431K mutant was expressed poorly in oocytes, and we were unable to observe single channels. The D431N mutant (which also changes the charge at this position, but by half as much) has a single-channel current-voltage curve indistinguishable from that of the wild type.
14. B. Frankenhaeuser, *J. Physiol. (London)* **152**, 159 (1960); F. J. Sigworth and B. C. Spalding, *Nature* **283**, 293 (1980); K. Imoto *et al.*, *ibid.* **335**, 645 (1988); R. MacKinnon, R. Latorre, C. Miller, *Biochemistry* **28**, 8092 (1989). Our reason for supposing that this site is located near the extracellular surface is the effect of mutations at this and nearby positions on CTX block (2).
15. W. Stühmer *et al.*, *EMBO J.* **8**, 3235 (1989).
16. G. C. Frech, A. M. J. Van Dongen, G. Schuster, A. M. Brown, R. H. Joho, *Nature* **340**, 642 (1989).
17. H. R. Guy, in *Monovalent Cations in Biological Systems*, C. A. Pasternak, Ed. (CRC Press, Cleveland, OH, 1989).
18. M. Noda, H. Suzuki, S. Numa, W. Stühmer, *FEBS Lett.* **259**, 213 (1990).
19. B. L. Tempel, D. M. Papazian, T. L. Schwarz, Y. N. Jan, L. Y. Jan, *Science* **237**, 770 (1987).
20. W. A. Catterall, *ibid.* **242**, 50 (1988).
21. The Shaker H4 cDNA clone (in a Bluescript vector) [A. Kamb, J. Tseng-Crank, M. A. Tanouye, *Neuron*

1, 421 (1988)] is nearly identical to Shaker B (19). Oligonucleotide-directed mutagenesis was performed with the *du<sup>r</sup> ung<sup>-</sup>* selection scheme of T. A. Kunkel [Proc. Natl. Acad. Sci. U.S.A. 82, 488 (1985)], with the Mutagene kit (Bio-Rad) and oligonucleotides (18 to 23 bp) with up to three mismatched bases (Amber Incorporated, Guilford, CT). After confirmation of the mutation by dideoxy sequencing of the mutated region [F. Sanger, S. Nicklen, A. R. Coulson, *ibid.* 74, 5463 (1977)] (Sequenase, U.S. Biochemical), RNA was prepared from the mutant DNA by linearization with Hind III (NEB) followed by *in vitro* transcription with T7 polymerase (Promega). *Xenopus* oocytes were prepared and injected as described previously [R. MacKinnon, P. Reinhart, M. M. White, *Neuron* 1,

997 (1988)]. To avoid misinterpretation resulting from stray mutations that might occur during the mutagenesis process, we confirmed results (that differed from wild-type properties) with two independently isolated mutant complementary DNAs.

22. F. Bezanilla and C. M. Armstrong, *J. Gen. Physiol.* 70, 549 (1977).

23. We thank C. Miller for helpful discussions and a critical review of the manuscript and T. Abramson and M. E. Jurman for technical assistance. The Shaker H4 cDNA clone was provided by R. J. Leonard and M. Tanouye. G.Y. is a Howard Hughes Medical Institute investigator. R.M. was supported by NIH research grant GM 43949.

11 May 1990; accepted 30 July 1990

## Neurotrophic and Neurotoxic Effects of Amyloid $\beta$ Protein: Reversal by Tachykinin Neuropeptides

BRUCE A. YANKNER,\* LAWRENCE K. DUFFY, DANIEL A. KIRSCHNER

**The amyloid  $\beta$  protein is deposited in the brains of patients with Alzheimer's disease but its pathogenic role is unknown. In culture, the amyloid  $\beta$  protein was neurotrophic to undifferentiated hippocampal neurons at low concentrations and neurotoxic to mature neurons at higher concentrations. In differentiated neurons, amyloid  $\beta$  protein caused dendritic and axonal retraction followed by neuronal death. A portion of the amyloid  $\beta$  protein (amino acids 25 to 35) mediated both the trophic and toxic effects and was homologous to the tachykinin neuropeptide family. The effects of the amyloid  $\beta$  protein were mimicked by tachykinin antagonists and completely reversed by specific tachykinin agonists. Thus, the amyloid  $\beta$  protein could function as a neurotrophic factor for differentiating neurons, but at high concentrations in mature neurons, as in Alzheimer's disease, could cause neuronal degeneration.**

**T**HE AMYLOID  $\beta$  PROTEIN IS A 39-TO 43-amino acid polypeptide that is the primary constituent of senile plaques and cerebrovascular deposits in Alzheimer's disease and Down syndrome (1). Although this protein has been well characterized biochemically, its primary biological function and role in the pathogenesis of Alzheimer's disease are unknown (2). Two seemingly disparate functions of amyloid  $\beta$  protein have been reported. A recombinant fragment of the amyloid precursor protein containing the amyloid  $\beta$  protein was neurotoxic to hippocampal neurons in culture (3). In another study, a peptide homologous to the amyloid  $\beta$  protein increased the survival of cultured hippocampal neurons (4). We now show that the amyloid  $\beta$  protein has both neurotrophic and neurotoxic effects that depend on neuronal age and the concentration of  $\beta$  protein.

We examined the effects of the amyloid  $\beta$  protein on neuronal viability in cultures of

hippocampal cells from rats at embryonic day 18 (E18). These cultures initially consist of undifferentiated round cells, 80 to 90% of which are neuronal precursors (5, 6). After 2 to 3 days in culture, 68 to 86% of these cells die and over 90% of the remaining cells begin to elaborate processes and acquire neuronal characteristics. Addition of a polypeptide corresponding to the first 40 amino acids of amyloid  $\beta$  protein ( $\beta$ 1-40) (7) to hippocampal cultures at the time of cell plating resulted in a significant increase in neuronal survival during the first 2 days when compared to control cultures (Fig. 1A). After 3 days in culture, however,  $\beta$ 1-40-treated cultures showed a marked decline in neuronal survival and by 4 to 5 days after plating the number of pyramidal neurons in  $\beta$ 1-40-treated cultures was significantly less than in control cultures (Fig. 1A).

When  $\beta$ 1-40 was added at the time of cell plating (day 0) there was a significant increase in neuronal survival 24 hours later relative to control cultures (Fig. 1, B and C). This trophic effect became progressively less apparent when  $\beta$ 1-40 was added during the next 2 days in culture. If  $\beta$ 1-40 was added to older cultures (3 days or more), it had a toxic effect on the neurons and caused a decline in neuronal survival 24 hours after

addition relative to controls (Fig. 1, B and C). After the initial period of cell death, control cultures showed only a small change in neuronal number from 2 to 5 days in culture. Thus,  $\beta$ 1-40 is neurotrophic during the early period of neuronal differentiation (days 0 to 2) when neuronal death and axonal outgrowth are occurring [stages 1 to 3 (8)]. Since mitosis of undifferentiated neuronal precursors is almost complete by E18 when the dissection is performed (8), the neurotrophic effect of  $\beta$ 1-40 is probably due to a decrease in neuronal death. In contrast,  $\beta$ 1-40 is neurotoxic to older, more differentiated neurons (days 3 to 5) that are undergoing dendritic growth and synaptogenesis [stages 4 and 5 (8)]. At this later stage of neuronal development,  $\beta$ 1-40 caused the almost complete collapse of the dendritic arbor, axonal retraction and thickening, and the appearance of vacuolar inclusions in the somatodendritic region (Fig. 1C). Glial cell number and morphology did not significantly change after addition of  $\beta$ 1-40 (9).

When  $\beta$ 1-40 was added to hippocampal cultures at a low concentration (0.1 nM), the early neurotrophic effect was observed but there was no neurotoxic effect, even after 3 days. We measured the  $\beta$ 1-40 concentration dependence of the neurotrophic and neurotoxic effects by adding  $\beta$ 1-40 at day 0 and day 4, respectively, and then determining neuronal survival after 24 hours (Fig. 2) (10). The half-maximal concentration for the trophic response was 0.06 nM, whereas the toxic response was first detected at 40 nM  $\beta$ 1-40 and the half-maximal concentration was about 100 nM. Thus, the trophic and toxic responses to  $\beta$ 1-40 depend on both the stage of neuronal differentiation and the concentration of  $\beta$ 1-40.

We determined the primary sequence of the  $\beta$ 1-40 domain responsible for the neurotrophic and neurotoxic effects by assaying overlapping peptides spanning the entire amyloid  $\beta$  protein sequence (Fig. 3 and Table 1). At 20  $\mu$ M,  $\beta$ 1-38 elicited the same activity as  $\beta$ 1-40.  $\beta$ 1-28 showed some activity but was much less potent than  $\beta$ 1-40 (Table 1).  $\beta$ 1-16 and  $\beta$ 17-28 showed no trophic or toxic activity at 20  $\mu$ M.  $\beta$ 17-28 showed similar activity to  $\beta$ 1-28 at higher concentrations (not shown). The  $\beta$ 25-35 peptide showed the same early neurotrophic and late neurotoxic activities as  $\beta$ 1-40.  $\beta$ 34-42 was inactive. A peptide corresponding to the COOH-terminal 20 amino acids of the amyloid precursor protein (APP 676-695) (11) and glucagon, a 28-amino acid peptide possessing  $\beta$ -pleated sheet structure similar to that of the amyloid  $\beta$  protein (12), were both inactive. Thus, the functional domain

B. A. Yankner and D. A. Kirschner, Department of Neurology, Harvard Medical School, Boston, MA 02115 and the Children's Hospital, Boston, MA 02115. L. K. Duffy, Department of Chemistry and Institute of Arctic Biology, University of Alaska, Fairbanks, AK 99775-0180.

\*To whom correspondence should be addressed.