Functional Consequences of Posttranslational Isomerization of Ser[46] in a Calcium Channel Toxin

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The venom of the funnel-web spider *Agelenopsis aperta* contains several peptides that paralyze prey by blocking voltage-sensitive calcium channels. Two peptides, ω-Aga-IVB (IVB) and ω-Aga-IVC (IVC), have identical amino acid sequences, yet have opposite absolute configurations at serine 46. These toxins had similar selectivities for blocking voltage-sensitive calcium channel subtypes but different potencies for blocking P-type voltage-sensitive calcium channels in rat cerebellar Purkinje cells as well as calcium-45 influx into rat brain synaptosomes. An enzyme purified from venom converts IVC to IVB by isomerizing serine 46, which is present in the carboxyl-terminal tail, from the L to the D configuration. Unlike the carboxyl terminus of IVC, that of IVB was resistant to the major venom protease. These results show enzymatic activities in *A. aperta* venom being used in an unprecedented strategy for coproduction of necessary neurotoxins that possess enhanced stability and potency.

Venomous animals have a variety of ingenious chemical strategies for predation. In some instances, peptide toxins are modified posttranslationally to enhance their stability and refine their biological activity (*1*). Many of these toxins are useful pharmacological tools, such as those targeting voltage-dependent Ca^{2+} channels (*2*, *3*). Studies of Ca^{2+} channels have focused on L-, N-, and P-type channels; however, the existence of additional subtypes has been shown by electrophysiology and molecular cloning (*4*). The spider toxin ω-Aga-IVA (IVA) (*5*), isolated from *A. aperta*, has aided in the classification of the P-type Ca^{2+} channel (*6*), which is insensitive to dihydropyridines (an L-type selective antagonist) and to ω-conotoxin GVIA (an N-type selective antagonist).

We report that *A. aperta* produces a related P-channel toxin, IVC, and uses a peptide isomerase to produce a third toxin, IVB, by inverting the stereochemistry at Ser[46] to the D configuration. Toxin peptide IVB is more potent than the L-serine counterpart, IVC, and appeared to be more stable when treated with an abundant (enkephalinase-like) neutral endopeptidase also purified from venom.

Purification of IVB and IVC followed a three-step procedure described in detail elsewhere (*7*). The toxins possess identical sequences (*8*), a COOH-terminal acid (*9*), and the same disulfide bonding pattern (*7*, *10*). Reversed-phase high-pressure liquid chromatography (rpHPLC) studies showed that the two toxin fractions were unique entities, because equimolar single injections (Fig. 1, A and C) or coinjections (Fig. 1B) provided well-resolved peaks. Molecular cloning of the gene encoding IVB and IVC was done from a complementary DNA (cDNA) library prepared from venom glands with the use of 3′ and 5′ rapid amplification of cDNA ends (RACE) techniques (*11*, *12*). Translation of this sequence confirms that the spider toxin is synthesized from a larger precursor molecule containing both a signal sequence for extracellular transport and a highly acidic propeptide sequence. Cloning results were in accord with chemical structure elucidation (*13*, *14*).

The fact that a D-amino acid was present in one of these toxins was found by treatment of each toxin with Glu-C (*7*). The NH_2-terminal native fragments in each case coeluted on HPLC, whereas the COOH-terminal peptides (Gly-Leu-Ser-Phe-Ala[48]) were easily distinguished. Pentapeptides containing both D- and L-serine were synthesized (*7*). Coinjection studies showed that the fragment generated from native IVC contains an L-serine residue and that the fragment from native IVB contains a D-serine residue (*15*).

Synthesis of the full-length toxins was required to rigorously establish the identity of the toxin diastereomers arising from D- and L-Ser[46]. Solid-phase synthesis and successful refolding (*16*) produced toxins that coeluted with their native counterparts and that were easily distinguished from the corresponding Ser[46] diastereomers when coinjected (*17*).

The most characteristic activity of the ω-Aga-IV class of toxins is a blockade of P-type Ca^{2+} channels in rat cerebellar Purkinje cells (*6*, *13*, *14*). An important difference in toxin potency was demonstrated for native IVB and IVC (*18*). A direct same-cell comparison of toxin activity showed that IVB blocks a greater portion of the

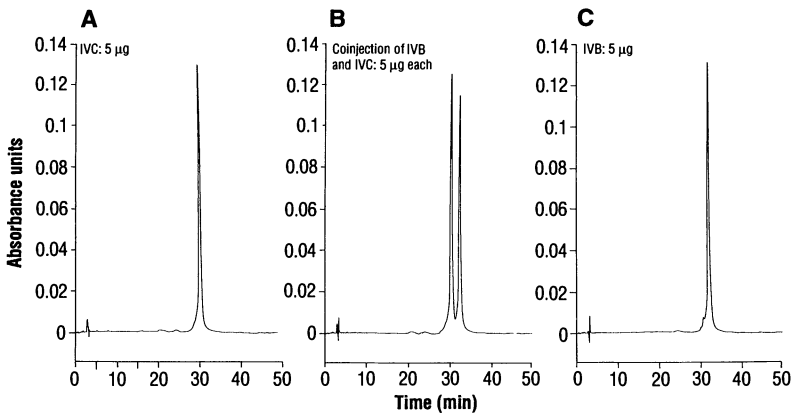

Fig. 1. Reversed-phase HPLC analysis and coinjection of IVB and IVC. Chromatography was done on a C-18 Vydac column (4.6 × 250 mm) with a particle size of 5 μm and a pore size of 300 Å. Flow rate was 1 ml/min.; detection occurred at 220 nm. The gradient was A = 0.1% TFA-H_2O, B = acetonitrile. The concentration of B was 25 to 35% over 40 min, then 35% for 10 min. Sample size: ~5 μg. (**A**) Response to a 5-μg injection of IVC. (**B**) Response to coinjection of IVB and IVC at 5 μg each. (**C**) Response to a 5-μg injection of IVB.

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current more rapidly at 32 nM than does IVC (Fig. 2). Dose-response studies showed an approximately fourfold difference in potency, with the more potent toxin bearing the D-Ser[46] residue [Fig. 2C; the inhibition concentration (IC_{50}) of IVB = 9.6 nM; IC_{50}(IVC) = 36.4 nM]. The predominant voltage-dependent Ca^{2+} channels found in rat brain synaptosomes, such as the P-channel in Purkinje cells, are refractory to the N-channel antagonist ω-conotoxin GVIA (<10% block at 1 μM; Fig. 3) and the L-channel antagonist nimodipine (17.3 ± 7.0% inhibition at 3 μM, mean ± SEM, n = 3) (*19*). Whereas 1 μM IVB maximally blocked 85% of the voltage-dependent flux, 3 μM IVC only blocked 67.5% of the flux. In a dose-response experiment, the D-Ser[46] analog displayed a fivefold greater potency than did the L-serine counterpart [IC_{50}(IVB) = 24.8 nM; IC_{50}(IVC) = 139 nM] (*20*). Electrophysiological recordings from rat cerebellar Purkinje cells that used synthetic toxins at 32 nM mirrored those made from cells treated with native toxins at similar concentrations (Fig. 2). Considering the variability of the recordings and the quantification of toxin peptides, a good correlation exists between the differences in potency and time course of the blockade of native toxins and those of synthetic toxins. These data further support the structure assignments given.

Crude *A. aperta* venom converted IVC to IVB. Isomerase activity was reduced by dilution of venom with phosphate-buffered saline (PBS) and was abolished by heat treatment (at 100°C for 30 min). Isomerization experiments using a crude size fraction (>10 kD) were difficult to interpret, because IVC was degraded at a rate comparable to that of isomerization. The enzyme predominantly responsible for the degradation of IVC was likely a neutral endopeptidase, because the proteolytic activity was greatly reduced by EDTA, reactivated by $ZnCl_2$, and inhibited by phosphoramidon; and it hydrolyzed small model peptides at a predicted enkephalinase cleavage site (*21*). Although isomerization could be carried out in the presence of EDTA, venom was fractionated on a Sephadex G-75sf gel column to purify the desired isomerase activity (*22*). Gel filtration was used to separate venom constituents, and SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 4) showed that fractionation of three major high molecular mass components was achieved. As was consistent with molecular mass markers, lanes 3, 7, and 9 (Fig. 4) contained proteins of mass 86,470 daltons, 36,589 daltons, and 29,469 daltons, respectively, as determined by mass spectrometry (*23*). No detectable enzymatic activity existed in fraction 7, whereas protease (*24*) and isomerase action were found in fractions 3 and 9, respectively. Fractions 9 to 11 were used to evaluate the isomerization of IVC to IVB and of IVB to IVC (*25*). Time course experiments (Fig. 5) qualitatively showed that the conversion of IVC to IVB was slightly faster than the reverse reaction, yet the reaction appeared to be rather slow in both directions. However, extended reactions times did produce a product ratio approaching 50:50. Enzyme and substrate concentrations in these experiments were ~2.3 and 15.8 μM, respectively, whereas the predicted concentrations in venom are ~42 and 750 μM, respectively. Therefore, one might expect the isomerization velocity in venom to be nearly 1000 times greater than was seen in these experiments.

The three-dimensional structure of IVB shows a cysteine-rich densely packed core with a flexible extended tail composed of the COOH-terminal 12 amino acids (*10*). An IVA congener isolated from venom lacking the final two amino acids was about six times less potent than was full-length toxin in blocking P-type channels in rat Purkinje cells. It is logical that this peptide arises from the proteolytic action of a neutral endopeptidase on IVA, because the five COOH-terminal amino acids found in this peptide are Gly-Leu-Gly-Leu-Ala[48]. Further demonstration of the importance of the COOH-

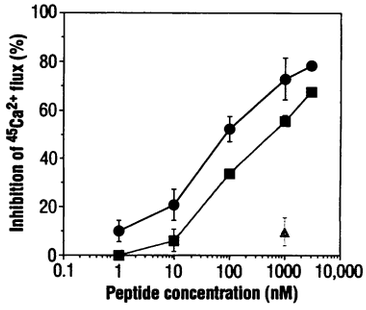

Fig. 3. Dose-response curves for the blockade of $^{45}Ca^{2+}$ flux by peptide toxins in rat brain synaptosomes. Synaptosomes were prepared as described in (*6*) and studies were carried out as described in (*20*). Circles, IVB; squares, IVC; and triangle, ω-conotoxin GVIA.

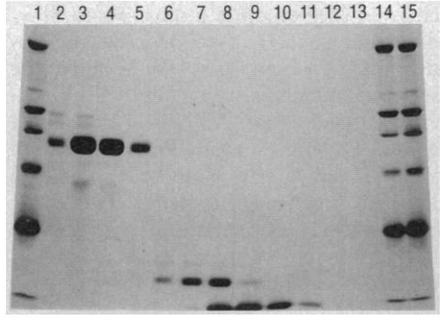

Fig. 4. Linear 5 to 15% SDS-PAGE gel. Lanes 2 to 13 are fractions taken from a G-75sf Sephadex column (see *22*). Lanes 2 to 5 contain purified protease (~80 kD) and lanes 9 and 10 contain purified racemase (~30 kD). Lanes 1, 14, and 15 are molecular mass standards; from top to bottom, they are myosin (200 kD), *Escherichia coli*–galactosidase (116 kD), rabbit muscle phorphylase b (97.4 kD), bovine serum albumin (66.2 kD), hen egg white ovalbumin (45 kD), and bovine carbonic anhydrase (31 kD).

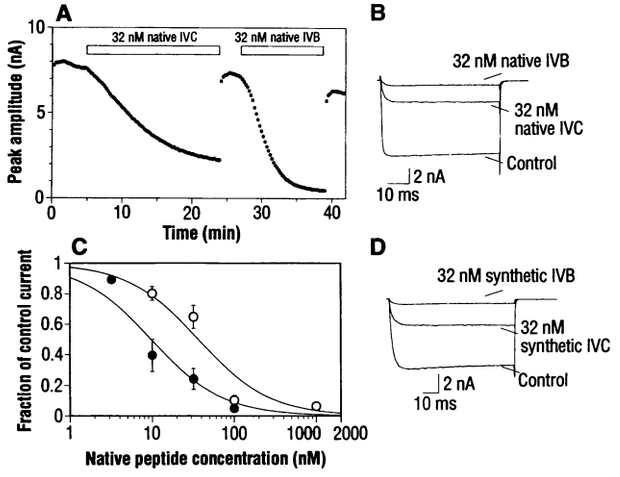

Fig. 2. Comparison of the block by IVB and IVC of P-type calcium currents. (**A**) Time course of the inhibition of P-channel currents in a single Purkinje cell. IVB blocked the current faster and more efficiently than did the same concentration of IVC during the peptide application periods shown by open bars (for 32 nM native IVB, inhibition = 81 ± 5% and τ_{block} = 7.6 ± 2.7 min; for 32 nM native IVC, inhibition = 39 ± 8% and τ_{block} = 32.8 ± 12.7 min for five same-cell experiments). Immediately after the cessation of peptide application, control medium was applied to the cell through microcapillary tubes, and a series of depolarizing steps was applied to enhance the rate of toxin washout (*5*). (**B**) Whole-cell currents from the experiment shown in (A). (**C**) Dose-response relation of the block by IVB (filled circles) and IVC (open circles) of high-threshold currents in rat Purkinje cells. The solid curves are best fit of $F = 1/(1 + [\text{peptide}]/IC_{50})$, with IC_{50} = 9.6 nM for IVB and 36.4 nM for IVC (F, fraction of control current). Each point represents the mean of data from five to eight cells. (**D**) Whole-cell currents from a cell treated with synthetic toxins (for 32 nM synthetic IVB, inhibition = 94 ± 0.6% and τ_{block} = 2.1 ± 0.6 min; for 32 nM synthetic IVC, inhibition = 58 ± 7.5% for five same-cell experiments and τ_{block} = 17.5 ± 2.9 min for the same-cell experiments).

terminal tail to activity was obtained by removal of the last eight or nine amino acids of IVB or IVC with thermolysin (*19*). Truncation produced toxins that were entirely devoid of P-channel activity in Purkinje cells. It can therefore be concluded that interaction of the COOH-terminal tail with the target channel is essential for high-potency blockade. The isomerase activity, which operates exclusively on Ser^{46}, protects the COOH-terminus of the toxin from degradation while affording a three- to fivefold increase in potency. Enhanced stability may explain why IVB is two to four times more abundant in venom than are the other toxins of its class.

Incorporation of D–amino acids into eukaryotic proteins is rare. Notable examples are the opioid peptides (such as dermorphins), which are found in the secretions of frog skin (*26*). Although it is apparent that an enzymatic activity is involved, the timing and mechanism by which the frog manipulates the configuration of a specific internal amino acid are not clear (*27*). The isomerase activity in *A. aperta* venom is distinguished from that of other cofactor-independent racemases (*28*, *29*). The spider isomerase operates on an intact peptide rather than on a free amino acid and may require specific flanking residues to operate efficiently. Therefore, in a strict sense the enzyme is not a racemase, because it catalyzes the interchange of two diastereomers, not enantiomers.

The favored mechanism for cofactor-independent racemases, which may be operative here, is a two-base model in which a pair of cysteine residues removes the α proton and subsequently reprotonates the intermediate from the opposite face (*28*, *29*). A different pathway might include dehydroalanine as the central intermediate in a diastereoselective dehydration-rehydration process. Alternatively, isomerization could proceed by means of a mechanism related to serine hydroxymethylase (*30*), in which the formaldehyde equivalent generated is not scavenged by tetrahydrofolate but instead is trapped by the reactive intermediate from the opposite face to produce the D configuration.

Neutral endopeptidases have been described in insects (*31*). Enkephalin immunoreactivity and specific binding in insect nervous tissue has been reported (*31*, *32*). Moreover, a role for enkephalins in modulating locomotor activity in invertebrates has been described (*33*). These data suggest a role for a soluble enkephalinase as a neurotoxic constituent in venom. Additionally, the endopeptidase could serve as an essential activity for toxin maturation or as a selection mechanism to enrich the venom in enkephalinase-resistant toxins produced by the serine isomerase. The isomerization of IVC to IVB not only enhances toxin stability to such protease activity but also confers increased potency at specified physiological targets. These toxins naturally target channels found in invertebrate tissue. Although the toxin activity measured here uses mammalian tissues, the first channel cloned from an invertebrate (*34*) is highly homologous to mammalian counterparts. Identification of the invertebrate channel subtype to which class IV toxins are directed will require additional cloning and functional expression.

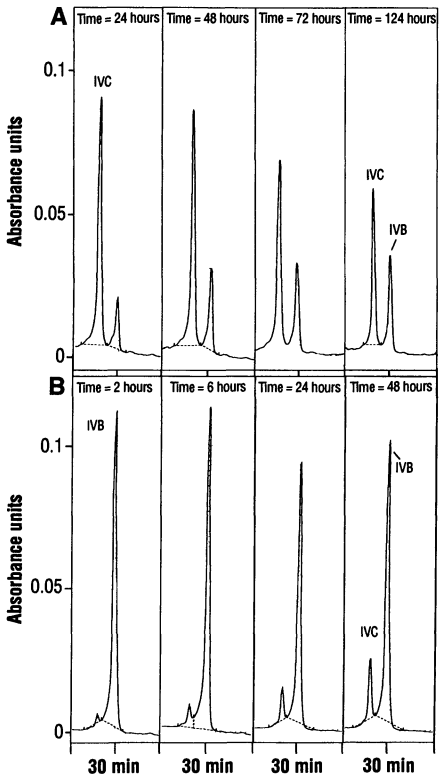

Fig. 5. (**A**) Isomerization of IVC to IVB. (**B**) Isomerization of IVB to IVC; 200 μl (derived from 20 μl of crude venom) of *A. aperta* fraction 9 (G-75sf Sephadex) in PBS (pH 7.4). Chromatography was done on a C-18 Vydac column (4.6 × 250 mm) with a particle size of 5 μm and a pore size of 300 Å. Flow rate was 1 ml/min; detection occurred at 220 nm. The gradient was A = 0.1% TFA-H_2O, B = acetonitrile. The concentration of B was 25 to 35% over 40 min, then 35% for 10 min. Time range: 23 to 35 min. Sample size: ~5 μg.

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7. S. D. Heck *et al.*, *J. Am. Chem. Soc.*, in press.
8. Structure assignments for IVB and IVC were made according to amino acid composition, sequence analysis, and mass spectral data by means of methods described in (*7*). Molecular mass data (5273.0 ± 0.4 daltons) for native peptides was consistent with the prediction made from determined sequences with four disulfide bonds (a mass of 5273.034 daltons calculated for $C_{215}H_{337}N_{65}O_{70}S_{10}$). Peptides were sequenced in native form and as *S*-pyridylethylated derivatives generated in situ. The free NH_2-terminus of each peptide was capped with acetic anhydride. Two COOH-terminal fragments, Ile^{30} to Ile^{41} and Glu^{43} to Ala^{48}, were generated in situ by treatment with cyanogen bromide and sequenced.
9. Cleavage of IVB and IVC at Met^{42} with CNBr releases the final six–amino acid fragment (Glu^{43} to Ala^{48}). Fast atom bombardment mass spectrometry (FAB-MS) analysis of this peptide assigned the COOH-termini as free acids. Mass ions for IVB (623.3 and 645.3 daltons) correspond to the COOH-terminal acid (Glu-Gly-Leu-Ser-Phe-Ala; $C_{28}H_{42}N_6O_{10}$) as $(M + H)^{1+}$ and $(M + Na)^{1+}$, respectively. Mass ions for IVC (623.3, 645.3, and 661.2 daltons) correspond to the COOH-terminal acid as $(M + H)^{1+}$, $(M + Na)^{1+}$, and $(M + K)^{1+}$, respectively. For CNBr cleavage, IVB or IVC (50 μg, 9.61 nmol) in 40 μl of 70% formic acid was treated with 10 μl of a 140 mM solution of cyanogen bromide in 70% formic acid. The reaction was kept dark at 24°C for 18 hours, diluted with 100 μl of water, lyophilized to dryness, and analyzed by FAB-MS.
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12. 3′ RACE yielded a 310–base pair (bp) product with the use of ethidium-stained 4% agarose gel. Subcloning and sequencing confirmed the amplification of the cDNA encoding IVB and IVC. The coding sequence and the 135-bp region preceding the polyadenylate [poly(A)] tail account for 282 bp of the sequence for the gene encoding IVB and IVC. A 280-bp 5′ RACE product was cloned into the plasmid encoding the cDNA sequence for the mature toxin. The resultant subclone represents the complete cDNA encoding IVB and IVC. For 3′ RACE, venom glands from *A. aperta* spiders were frozen in liquid N_2. RNA was extracted from the glands [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)] and polyadenylate mRNA was purified with oligo(dT) cellulose and reverse-transcribed to cDNA with murine leukemia virus reverse transcriptase and a Not I primer-adapter. An oligonucleotide corresponding to residues 1 through 8 of IVB and IVC was designed with the use of *Drosophila* codons. The sequence of the 5′ oligo was: 5′-GAGGA C/TAAC/TTGCATT/CGCNGAG/AGA. Each oligonucleotide was used in the polymerase chain reaction (PCR) at 2 μM, with 10 to 100 ng of dT-primed cDNA in a 50-μl reaction volume. The reaction was heated to 95°C for 2 min, followed by annealing of the primers for 2 min at 37°C and then extension of the primers at 72°C for 1 min. This cycle was repeated twice and then switched to an identical profile that used an elevated annealing temperature of 54°C. This program was repeated 35 times. The single PCR product, visualized on an ethidium-stained 3% NuSieve–1% SeaKem agarose gel, was purified through a Centricon-100 filter. The insert, digested with Not I, was cloned into a pBluescript KS vector that had been previously digested with Eco RV and Not I. The inserts of three subclones were then sequenced. For 5′ RACE, strand cDNA prepared as described above was tailed at the 3′ end with dG residues by means of terminal deoxynucleotide transferase. The cDNA sequences upstream of the sequences encoding the mature toxin were amplified by PCR with a gene-specific internal oligonucleotide (amino acids 30 to 24) and a dC-tailed anchored primer with the use of a temperature profile of 2 min at 94°C, 2 min at 37°C, and 1 min at 37°C. This cycle was repeated twice,

and the program was switched to an identical profile incorporating an elevated annealing temperature of 54°C and was repeated 35 times. The amplification product was digested with the enzymes Pst I and Sal I and purified from an agarose gel with the glassmilk resin supplied in the GeneClean kit. The plasmid containing the mature toxin was digested with the same enzymes to create a vector for directional cloning. Subclones were screened by DNA sequencing. The GenBank accession number for the gene encoding IVB and IVC is U15925.

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14. The sequence and primary activity of IVB and IVC are available (*13*) [M. E. Adams *et al.*, *Mol. Pharmacol.* **44**, 681 (1993); T. Teramoto *et al.*, *Biochem. Biophys. Res. Commun.* **196**, 134 (1993)]. The presence of two toxins with identical primary structures was reported more recently [A. H. Ganong *et al.*, *Soc. Neurosci. Abstr.* **19**, 7212 (1993)].
15. A library of all diastereomers was synthesized to verify that the chromatography used to distinguish the native proteolytic fragments could resolve all possible isomers of this peptide. The proteolytic products Gly-Leu-L-Ser-Phe-Ala and Gly-Leu-D-Ser-Phe-Ala from native venom each independently coeluted with one of the synthesized peptide diastereomers (*7*).
16. Peptide synthesis was done on an ABI 430A (Applied Biosystems, Foster City, CA) with the use of *tert*-butoxycarbonyl protocols (version 1.4; 1-methyl-2-pyrrolidinone and hydroxybenzotriazole). Capping was done after each coupling step. Butoxycarbonyl (BOC) AlaPAM (0.66 g, 0.753 mmol/g) resin and standard side-chain–protected BOC amino acids were used in chain assembly. Hydrofluoric acid (HF) deprotection and cleavage from resin (500 mg) followed the low-high method of J. Tam and colleagues [*J. Am. Chem. Soc.* **105**, 6442 (1983)]. The crude HF product (262 mg) was stirred into 1 liter of 0.4% trifluoroacetic acid (TFA) for 1 hour, then pH-adjusted to 8.0 with NH_4OH and filtered with a 0.22-μm filter. The solution was swirled once daily. Reversed-phase HPLC showed that a major product developed in 8 to 12 days. Active toxin was purified from the refolding by means of reversed-phase chromatography on a POROS R2M column (25.4 × 100 mm) by ion exchange on a polysulfoethylaspartamide column (4.6 × 200 mm), and by rpHPLC on a C-18 Vydac column (4.6 × 250 mm). Synthetic IVB and IVC were shown to be identical to the corresponding native toxins in all respects.
17. Chromatography was done as a C-18 Vydac column (4.6 × 250 mm), with a particle size of 5 μm and a pore size of 300 Å. Flow rate was 1 ml/min; detection occurred at 220 nm. The gradient was A = 0.1% TFA, B = acetonitrile. The concentration of B was 25 to 35% over 40 min, then 35% for 10 min. Results obtained were identical to those outlined in Fig. 1, A through C.
18. Animals used in these studies were treated in strict accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (NIH Publ. no. 85-23, U.S. Department of Health and Human Services, Public Health Department, Bethesda, MD, 1985). Freshly dissociated cerebellar Purkinje neurons from rats 7 to 15 days postnatal were prepared by means of a modification of described methods [L. J. Regan, *J. Neurosci.* **11**, 2259 (1991); J. E. Huettner and R. W. Baughman, *ibid.* **6**, 3044 (1986)]. Whole-cell recordings of P-type currents (carried by Ba^{2+}) were recorded at room temperature with 1- to 3-megohm patch electrodes containing 90 mM CsOMs, 18 mM CsF, 9 mM EGTA, 9 mM Hepes, 4 mM $MgCl_2$, 14 mM creatine phosphate (2 Na), 1 mM tris–guanosine triphosphate, and 4 mM Mg–adenosine triphosphate, adjusted to pH 7.3 with CsOH and to 325 mosM with sucrose. The extracellular buffer for seal formation contained 150 mM NaCl, 10 mM Hepes, 4 mM KCl, 2 mM $CaCl_2$, 3 mM $BaCl_2$, 2 mM $MgCl_2$, and 10 mM deoxyglucose at pH 7.4. After a seal was obtained and whole-cell recording was done, the extracellular medium was switched to 154 mM tetraethylammonium chloride, 10 mM Hepes, 5 mM $BaCl_2$, 2 mM $MgCl_2$, 10 mM deoxyglucose, and 0.25 mM tetrodotoxin, adjusted to pH 7.4 and to 335 mosM. Series resistance was compensated by 80%. Cells were voltage-clamped at −80 mV, and maximum inward barium currents were induced by a 50-ms depolarizing step to −10 to +10 mV. Leak current was subtracted with four scaled hyperpolarizing pulses stepped from a holding potential of −80 mV. Test solutions were delivered through 152-μm microcapillary tubes by gravity feed. Toxin peptide solutions contained cytochrome c (1 mg/ml). Currents recorded under these conditions were blocked 95 ± 1% (n = 8) by 200 nM synthetic IVA and were blocked 6 ± 1% (n = 9) by a combination of 3.2 μM ω-conotoxin GVIA and 10 μM nifedipine.
19. S. D. Heck *et al.*, data not shown.
20. $^{45}Ca^{2+}$ flux into rat brain synaptosomes was done essentially as described in (*5*). Synaptosomes were preincubated with toxin for 30 min (20 min at 4°C and 10 min at 30°C) before depolarization with 45 mM KCl in the presence of 0.5 μCi of $^{45}Ca^{2+}$. The $^{45}Ca^{2+}$ flux was 0.053 ± 0.015 nmol per milligram of protein. Each concentration data point represents the mean ± SEM for at least three determinations except where noted.
21. Partial sequence data has been reported [fraction M in (*13*)].
22. Venom (~100 μl) was diluted with PBS (100 μl), loaded on a G-75sf gel column (10 mm inside diameter × 10 cm), and eluted with PBS at 400 μl/min, collecting 1.4-ml fractions.
23. Electrospray mass spectrometry data was collected as described in (*7*).
24. The COOH-terminal five–amino acid peptides of IVB and IVC (Gly-Leu-DLSer-Phe-Ala) were used as model substrates to study the proteolytic activity in fractions 3 and 4. The COOH-terminal five–amino acid peptide of IVB, containing a D-serine residue, was completely resistant to proteolysis and retarded the cleavage of the analogous L-amino acid substrate derived from IVC. The protease was isolated from venom and shows a high homology to neutral endopeptidase class 24.11 (lanes 3 and 4, Fig. 4). Alignment with related proteases places residue 1 of this enzyme with residue 53 of homologs. The extremely hydrophobic NH_2-terminus in the later enzymes is suggested to serve as a membrane anchor. The spider protease lacking this element may be designed as a soluble protein either for injection into prey or to serve as a necessary protease for toxin selection or maturation.
25. Gel fractions 9, 10, and 11 were combined, lyophilized, and reconstituted in 500 μl of water. To 200 μl of this sample was added 20 μl of 50 mM EDTA and 20 μg of IVC or IVB as a solution in 20 μl of water. The sample was kept at 37°C for the duration of the experiment.
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35. The authors acknowledge the contributing efforts of L. H. Hirning, A. M. Mueller, L. D. Artman, J. L. Ives, J. A. Lowe, E. F. Nemeth, K. F. Geoghegan, G. O. Daumy, T. J. Carty, J. G. Stroh, C. Drong, K. Rosnack, and F. Fuller.

27 June 1994; accepted 5 October 1994

Spermine and Spermidine as Gating Molecules for Inward Rectifier K^+ Channels

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Inward rectifier K^+ channels pass prominent inward currents, while outward currents are largely blocked. The inward rectification is due to block by intracellular Mg^{2+} and a Mg^{2+}-independent process described as intrinsic gating. The rapid loss of gating upon patch excision suggests that cytoplasmic factors participate in gating. "Intrinsic" gating can be restored in excised patches by nanomolar concentrations of two naturally occurring polyamines, spermine and spermidine. Spermine and spermidine may function as physiological blockers of inward rectifier K^+ channels and "intrinsic" gating may largely reflect voltage-dependent block by these cations.

Gating of voltage-dependent ion channels is generally thought to be an intrinsic property of the channel protein (*1*). However, gating may be specifically modified by accessory proteins (β subunits) (*2*), small organic molecules (for example, adenosine triphosphate) (*3*), or inorganic cations such as Ca^{2+} or Mg^{2+} (*4*–*6*). The intrinsic gating of inward rectifier K^+ channels (IRKs) (*6*, *7*) is thought to contribute to inward rectification by producing closure of channels at depolarized potentials, resulting in negligible outward currents even in the absence of intracellular Mg^{2+} (Mg_i^{2+}). Conversely, reopening of channels at hyperpolarized potentials is thought to cause the time-dependent onset of inward currents. Two IRKs, IRK1 (*8*) and ROMK1 (*9*), ex-

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