Takio, K., Titani, K., Ericsson, L. H., & Yonetani, T. (1980) *Arch. Biochem. Biophys. 203*, 615-629.
Tollin, G., Hanson, L. K., Caffrey, M., Meyer, T. E., & Cusanovich, M. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3693-3697.
Waldmeyer, B., Bechtold, R., Bosshard, H. R., & Poulos, T. L. (1982) *J. Biol. Chem. 257*, 6073-6076.
Wittenberg, B. A., Kampa, L., Wittenberg, J. B., Blumberg, W. E., & Peisach, J. (1968) *J. Biol. Chem. 243*, 1863-1870.
Yamada, H., Makino, R., & Yamazaki, I. (1975) *Arch. Biochem. Biophys. 169*, 344-353.
Yandell, J. K., & Yonetani, T. (1983) *Biochim. Biophys. Acta 748*, 263-270.
Yonetani, T. (1971) in *Probes of Structure and Function of Macromolecules and Membranes II* (Chance, B., Yonetani, T., & Mildvan, A., Eds.) pp 545-552, Academic, New York.
Yonetani, T., & Ray, G. S. (1965) *J. Biol. Chem. 240*, 4503-4508.
Yonetani, T., & Ray, G. S. (1966) *J. Biol. Chem. 241*, 700-706.
Yonetani, T., & Anni, H. (1987) *J. Biol. Chem. 262*, 9547-9554.
Yonetani, T., Schleyer, H., & Ehrenberg, A. (1966a) *J. Biol. Chem. 241*, 3240-3243.
Yonetani, T., Wilson, D. F., & Seamonds, B. (1966b) *J. Biol. Chem. 241*, 5347-5352.
Yonetani, T., Yamamoto, H., Erman, J. E., Leigh, J. S., & Reed, G. H. (1972) *J. Biol. Chem. 247*, 2447-2455.

A Novel Sodium Channel Inhibitor from *Conus geographus*: Purification, Structure, and Pharmacological Properties†

Yuchio Yanagawa,*,‡ Teruo Abe,‡ Mei Satake,‡ Shoji Odani,§ Junichi Suzuki,∥ and Kiichi Ishikawa∥

Department of Neurochemistry, Brain Research Institute, and Department of Biochemistry, School of Medicine, Niigata University, Niigata, Japan, and Department of Biochemistry, School of Medicine, Yamagata University, Yamagata, Japan

Received October 15, 1987; Revised Manuscript Received May 5, 1988

ABSTRACT: A novel toxin, tentatively named conotoxin GS (CGS), has been isolated from a marine snail, *Conus geographus*. CGS was found to exist as a single polypeptide chain, consisting of 34 amino acid residues, cross-linked by three disulfide bonds. Its amino acid sequence was shown to be Ala-Cys-Ser-Gly-Arg-Gly-Ser-Arg-Cys-Hyp-Hyp-Gln-Cys-Cys-Met-Gly-Leu-Arg-Cys-Gly-Arg-Gly-Asn-Pro-Gln-Lys-Cys-Ile-Gly-Ala-His-Gla-Asp-Val. In competition experiments, CGS inhibited the bindings of [^{3}H]Lys-tetrodotoxin ([^{3}H]Lys-TTX) and [^{3}H]propionylconotoxin GIIIA to *Electrophorus electricus* electroplax membranes, with K_i values of 34 nM and 24 nM, respectively. The toxin inhibited the binding of [^{3}H]Lys-TTX (1 nM) to rat skeletal muscle homogenates with an IC_{50} value of 880 nM but showed very little effect on this binding to the rat brain P_2 fraction at 10 μM. These binding studies indicate that CGS belongs to the same group of Na channel inhibitors as TTX, STX (saxitoxin), and μ-conotoxins. Although CGS, like the μ-conotoxins, is a pharmacological probe for distinguishing between neuronal and muscle Na channel subtypes, the homology in the sequences of CGS and μ-conotoxins is very limited.

The voltage-dependent Na channel is the target of a variety of neurotoxins (Narahashi, 1974; Catterall, 1980, 1986). These toxins are useful in investigations on the structural and functional domains of the Na channel at the molecular level (Catterall, 1985). Studies have shown that different isoforms or subtypes of Na channels are present in various tissues and have indicated that these toxins are also valuable in studying heterogeneity of Na channels (Barchi, 1987). The class of neurotoxins that includes tetrodotoxin (TTX)[1] and saxitoxin (STX) is water-soluble heterocyclic guanidinium compounds which inhibit ion transport through Na channels by binding highly specifically to these channels. Two classes of Na channels differing in sensitivities to TTX have been described as TTX-sensitive and TTX-insensitive Na channels, and there are reports of the coexistence of these two types of Na channels in primary cultures of rat myoblasts and myotubes (Gonoi et al., 1985; Weiss & Horn, 1986) and in denervated muscles of adult rats (Pappone, 1980).

The venom of the marine snail *Conus geographus* contains a number of peptide toxins with various biological activities (Olivera et al., 1985). For example, ω-conotoxins inhibit Ca channels at the presynaptic terminus, α-conotoxins block nicotinic acetylcholine receptors at the postsynaptic membranes, and μ-conotoxins inhibit Na channels in the muscle.

μ-Conotoxins are basic peptides, each composed of 22 amino acid residues (Sato et al., 1983; Cruz et al., 1985). These peptide toxins can distinguish not only between neuronal and

† This study was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture.

* Correspondence should be addressed to this author.
‡ Department of Neurochemistry, Brain Research Institute, Niigata University.
§ Department of Biochemistry, School of Medicine, Niigata University.
∥ Department of Biochemistry, School of Medicine, Yamagata University.

[1] Abbreviations: TTX, tetrodotoxin; STX, saxitoxin; [^{3}H]Lys-TTX, [^{3}H]Lys-tetrodotoxin; [^{3}H]Pr-CGIIIA, [^{3}H]propionylconotoxin GIIIA; CGS, conotoxin GS; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; Gla, 4-carboxyglutamic acid; DTT, dithiothreitol; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; CamCys, carbamoylcysteine; Hyp, 4-hydroxyproline; CGIIIB, conotoxin GIIIB.

0006-2960/88/0427-6256$01.50/0

muscle Na channels (Cruz et al., 1985; Kobayashi et al., 1986) but also between TTX-sensitive and TTX-insensitive muscle Na channels (Gonoi et al., 1987). They preferentially block the adult skeletal muscle Na channel in a similar way to guanidinium toxins but have no significant effects on Na channels in nerves (e.g., brain, peripheral nerve, and giant axon). In addition, direct ligand binding studies with [^{3}H]-Lys-TTX, [^{3}H]STX, or [^{3}H]Pr-CGIIIA indicate that μ-conotoxins and guanidinium toxins bind with similar affinities to a commun receptor site on Na channels in adult skeletal muscle and the eel electroplax but that μ-conotoxins do not effectively block the binding of radiolabeled TTX and STX to Na channels in nerves (Moczydlowski et al., 1986; Ohizumi et al., 1986; Yanagawa et al., 1986, 1987).

During preparation of μ-conotoxins, we found a novel peptide toxin, tentatively designated as conotoxin GS (CGS). CGS is composed of 34 amino acid residues including the unusual amino acids 4-hydroxyproline and 4-carboxyglutamic acid. Pharmacological binding studies showed that CGS inhibited the bindings of both [^{3}H]Lys-TTX and [^{3}H]Pr-CGIIIA to *Electrophorus electricus* electroplax membranes and the binding of [^{3}H]Lys-TTX to rat skeletal muscle but had very little effect on the binding of [^{3}H]Lys-TTX to rat brain. This paper describes the purification, structure, and pharmacological properties of this novel conotoxin from *C. geographus* venom.

Materials and Methods

Purification of the Novel Conotoxin CGS. The venom ducts from *C. geographus* were homogenized, and soluble materials were fractionated on a column of Sephadex G-25 as described by Yanagawa et al. (1987). Fractions I–V were obtained by monitoring absorbance at 254 nm. Of these fractions, fraction II, containing the Na channel inhibitors, was lyophilized and further fractionated by reverse-phase HPLC as follows: Fraction II (32 mg) was dissolved in 1.4 mL of 0.1% TFA (solution A), applied to a Waters μBondapak C18 column (1.9 × 15 cm), and eluted with a 40-min linear gradient of 14–30% solution B (60% acetonitrile in 0.1% TFA) in solution A. Fractions were collected manually, lyophilized, and subjected to amino acid analysis. Aqueous solutions of 10–100 μM CGS were stored in polyethylene tubes, and more dilute solutions were made in 0.5% (w/v) BSA and 0.05% (w/v) NaN_3 to prevent loss of the basic peptide toxin.

Amino Acid Analysis. Peptide samples were hydrolyzed with 5.7 N HCl in vacuo at 110 °C for 20 h, and the amino acid compositions of the hydrolysates were determined in a Hitachi amino acid analyzer (Model 835). Methionine and cystine were determined after performic acid oxidation (Hirs, 1967). The Gla standard was obtained from Sigma. The relative ninhydrin color factor for Gla is 38% of that for glutamic acid. Gla was analyzed after hydrolysis at 110 °C for 22 h in 2.0 N KOH (McIntosh et al., 1984).

Chemical Modification of Disulfide Bonds. Disulfide bonds were cleaved by reduction with DTT followed by S-pyridylethylation with 4-vinylpyridine (Hermodson et al., 1973) and S-carbamoylmethylation with iodoacetamide (Ui, 1979), respectively. The excess reagents and salts were removed by reverse-phase HPLC on a Waters Radial-Pak C18 column (0.8 × 15 cm).

Enzymatic Digestion. The reduced and S-pyridylethylated sample (7 nmol) was digested with TPCK-trypsin (5 μg) in 200 μL of 10 mM NH_4HCO (pH 8.0) for 3 h at 37 °C, and the reaction mixture was lyophilized for desalting.

Peptides obtained by tryptic digestion were separated by reverse-phase HPLC as follows: The lyophilized material was dissolved in 0.1% TFA and applied to a Toyo Soda ODS (octadecylsilica) 120T column (0.45 × 25 cm) in a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 mL/min. A Hitachi 638-30 liquid chromatograph was used.

Sequence Analysis. Automated Edman degradation of the peptides was performed on an Applied Biosystems 470A gas-phase sequenator.

Preparation of Eel Electroplax Membranes, the Rat Brain P_2 Fraction, and Rat Skeletal Muscle Homogenates. Electroplax membranes from the electric eel *Electrophorus electricus* were prepared as described by Yanagawa et al. (1986). A whole rat brain was homogenized in 10 volumes of 0.32 M sucrose in 10 mM Hepes (adjusted to pH 7.4 with Tris base) containing 0.1 mM PMSF and 1 mM iodoacetamide. The resulting homogenate was centrifuged at 1000g for 10 min. The supernatant was centrifuged at 28000g for 60 min, and the P_2 fraction was obtained as a pellet.

Rat hind leg and back muscle were minced and homogenized in 7.5 volumes of 140 mM choline chloride and 20 mM Hepes–Tris (pH 7.4) with a polytron. The homogenate was filtered through a nylon mesh (pore size, 106 μm) to remove connective tissue and then used for binding studies.

Binding Experiment. [^{3}H]Lys-TTX (30 Ci/mmol) and [^{3}H]Pr-CGIIIA (105 Ci/mmol) were synthesized as described by Chicheportiche et al. (1980) and Yanagawa et al. (1987), respectively. Protein was measured by the method of Lowry et al. (1951) with BSA as a standard.

Eel electroplax membranes (30–60 μg of protein/mL) were incubated at 4 °C in 2 mL of incubation medium consisting of 40 mM choline chloride, 0.1% (w/v) BSA, 0.01% (w/v) NaN_3, 20 mM Tris-HCl (pH 7.4), and an appropriate concentration of [^{3}H]Pr-CGIIIA or [^{3}H]Lys-TTX.

Rat skeletal muscle homogenates (13 mg wet wt/mL) and brain P_2 fraction (100–180 μg of protein/mL) were incubated at 4 °C in 1 mL of incubation medium consisting of 140 mM choline chloride, 0.1% (w/v) BSA, 0.01% (w/v) NaN_3, 20 mM Hepes–Tris (pH 7.4), and an appropriate concentration of [^{3}H]Pr-CGIIIA or [^{3}H]Lys-TTX.

After incubation, samples were centrifuged at 28000g for 15 min at 0 °C. The supernatant fluids were decanted, and the pellets obtained were solubilized in 1 mL of 0.4% SDS in 0.4 N NaOH and neutralized with 5 N HCl. After addition of 9 mL of ACS-II (Amersham), radioactivity was measured in a Beckman liquid scintillation counter. Nonspecific binding was determined in the presence of unlabeled CGIIIA (1–2 μM) or unlabeled TTX (1–2 μM), and specific binding was calculated by subtracting nonspecific binding from total binding.

In competition experiments, various concentrations of the competing neurotoxins were incubated under the same conditions as described above with the eel electroplax membranes or rat skeletal muscle homogenates or rat brain P_2 fraction and 1 nM [^{3}H]Lys-TTX or 1 nM [^{3}H]Pr-CGIIIA.

For each toxin, three to five experiments were performed. Half-maximal inhibition of specific binding gave IC_{50} values from which inhibition constants (K_i) were calculated as

$$K_i = \frac{IC_{50}}{1 + [\text{toxin}]/K_d}$$

where [toxin] is the concentration of [^{3}H]Lys-TTX or [^{3}H]-Pr-CGIIIA and K_d is the dissociation constant of this toxin (Hollenberg, 1985).

Toxins and Chemicals. Citrate-free TTX was obtained from Sankyo, Tokyo. Saxitoxin was a generous gift from Dr. Y. Kishi. TPCK-trypsin and 4-vinylpyridine were from

Table I: Amino Acid Composition of Conotoxin GS[a]

amino acid	calculated	amino acid	calculated
Gla	0.79[b] (1)	Met[d]	0.90 (1)
Asp	2 (2)	Ile	1.00 (1)
Ser	2.04 (2)	Leu	1.15 (1)
Glu	2.00[b] (2)	Lys	1.19 (1)
Pro	1.08 (1)	His	0.97 (1)
Gly	6.14 (6)	Arg	3.92 (4)
Ala	2.13 (2)	Hyp	1.78 (2)
$^1/_2$-Cys[c]	6.15 (6)	total residue	34
Val	1.05 (1)		

[a] Values are expressed as molar ratios, normalizing Asp to the nearest integer. Figures in parentheses are numbers of residues determined from sequence analysis. [b] Determined by alkaline hydrolysis. [c] Measured as cysteic acid. [d] Measured as methionine sulfone.

Table II: Sequence Analysis of Conotoxin GS (1.5 nmol)

cycle	amino acid	yield (pmol)	cycle	amino acid	yield (pmol)
1	Ala	758	18	Arg	115
2	PeCys[a]	NQ[b]	19	PeCys	NQ
3	Ser	307	20	Gly	160
4	Gly	338	21	Arg	161
5	Arg	250	22	Gly	134
6	Gly	259	23	Asn	108
7	Ser	235	24	Pro	115
8	Arg	223	25	Gln	82
9	PeCys	NQ	26	Lys	29
10	Hyp	NQ	27	PeCys	NQ
11	Hyp	NQ	28	Ile	79
12	Gln	151	29	Gly	77
13	PeCys	NQ	30	Ala	79
14	PeCys	NQ	31	His	22
15	Met	170	32	X[c]	
16	Gly	163	33	Asp	41
17	Leu	146	34	Val	24

[a] *S*-(Pyridylethyl)cysteine. [b] Not quantified. [c] Not identified.

Worthington Biochemicals and Wako Pure Chemical Industries, respectively.

Results and Discussion

Preparation and Purity of CGS. The soluble venom from *C. geographus* was fractionated into five distinct fractions on a Sephadex G-25 column (Yanagawa et al., 1987). Fraction II, containing Na channel inhibitors, was further separated by reverse-phase HPLC (Figure 1). Peak a was composed of μ-conotoxins GIIIA and GIIIB (Cruz et al., 1985), which

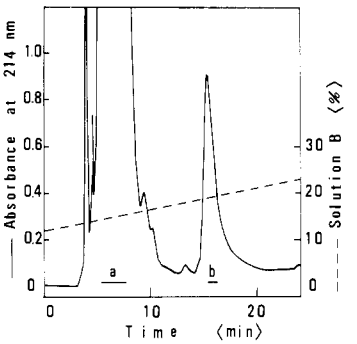

FIGURE 1: Last step of purification of conotoxin GS. This step was performed by reverse-phase HPLC. Lyophilized fraction II (32 mg) obtained by Sephadex G-25 gel filtration was dissolved in 1.4 mL of 0.1% TFA (solution A) and applied to a column (1.9 × 15 cm) of μBondapak C18 (Waters). Toxins were eluted with a 40-min linear gradient of 14–30% solution B (60% CH_3CN and 0.1% TFA) in solution A at a flow-rate of 10 mL/min.

were verified by amino acid analysis. Peak b was a single sharp peak of the novel Na channel inhibitor, tentatively named conotoxin GS (CGS). Usually, about 600 μg of CGS was isolated from the lyophilized extract of the soluble venom (425 mg), but the yield varied greatly with different batches of venom. Similar variation has been observed in the yields of other conotoxins (Gray et al., 1981; Cruz et al., 1985). The amino acid composition of the peptide after the final purification step was in complete agreement with the composition determined by sequencing (Table I).

Identification of Gla in CGS. Amino acid analysis of the acid hydrolysate of CGS indicated the presence of three residues of Glu per molecule. Direct automatic sequence analysis of S-pyridylethylated CGS gave the sequence from position 1 to position 34 except for position 32 (Table II), which was later shown to be Gla. S-Pyridylethylated CGS was digested with trypsin, and the resulting peptide mixture was separated by reverse-phase HPLC (Figure 2). Six major peptides, fragments 1–6 (6a and 6b), were obtained, and the amino acid compositions of their acid hydrolysates are given in Table III. The analyses showed little significant difference between fragments 6a and 6b. The amino acid compositions of all these tryptic peptides except for fragment 6 are consistent with the sequence of the whole toxin established directly with a sequenator.

Sequence analysis of tryptic fragment 6b provided the sequence PeCys-Ile-Gly-Ala-His-X-Asp-Val (Table IV). Again,

Table III: Amino Acid Compositions of Tryptic Fragments of Conotoxin GS[a]

	fragment						
	1 (residues 1–5)	2 (residues 6–8)	3 (residues 9–18)	4 (residues 19–21)	5 (residues 22–26)	6a (residues 27–34)	CGS
Asp					1.0 (1)	1.2 (1)	2
Ser	1.0 (1)	1.0 (1)					2
Glu			1.3 (1)		1.1 (1)	1.1 (1)	3
Pro					0.9 (1)		1
Gly	1.2 (1)	1.1 (1)	1.4 (1)	1.0 (1)	1.1 (1)	1.3 (1)	6
Ala	1.0 (1)					0.8 (1)	2
Val						0.9 (1)	1
Met			0.8 (1)				1
Ile						1.3 (1)	1
Leu			1.4 (1)				1
Lys					1.0 (1)		1
His						0.9 (1)	1
Arg	1.0 (1)	1.0 (1)	0.9 (1)	1.0 (1)			4
PeCys[b]	0.9 (1)		2.8 (3)	1.0 (1)		1.0 (1)	6
Hyp			1.7 (2)				2
total	5	3	10	3	5	8	34

[a] Amino acids were determined in acid hydrolysates. [b] *S*-(Pyridylethyl)cysteine.

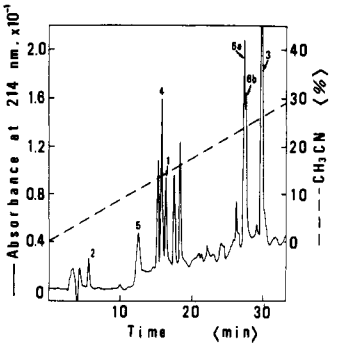

FIGURE 2: Separation of tryptic digest of S-pyridylethylated conotoxin GS by reverse-phase HPLC. The digest dissolved in 0.1% TFA was applied to an ODS column (0.45 × 25 cm) and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 mL/min. The indicated peaks of peptides were collected manually.

Table IV: Sequence Analysis of Carboxyl Region (Fragment 6b) of Conotoxin GS (0.3 nmol)

cycle	amino acid	yield (pmol)	cycle	amino acid	yield (pmol)
1	PeCys[a]	NQ[b]	5	His	34
2	Ile	86	6	X[c]	
3	Gly	81	7	Asp	43
4	Ala	79	8	Val	19

[a] *S*-(Pyridylethyl)cysteine. [b] Not quantified. [c] Not identified.

no identifiable PTH derivative was obtained at position 32, but tryptic fragment 6a was found to have one residue of Glu per fragment by amino acid analysis of the acid hydrolysate. We supposed that X at position 32 was probably Gla, because Gla is quantitatively decarboxylated to Glu during acid hydrolysis but is stable on alkaline hydrolysis (Price, 1983). Moreover, a PTH derivative of Gla cannot be identified by automatic Edman degradation (Hauschka et al., 1982; McIntosh et al., 1984).

Accordingly, we carried out alkaline hydrolysis of CGS. The amino acid composition of CGS is shown in Table I. As expected, CGS had one residue of Gla per molecule, as estimated from the difference of two and three glutamic acid residues recovered in alkaline and acid hydrolysates, respectively. Thus we concluded that position 32 was Gla.

Two residues of Hyp per molecule were identified by chromatography of the free amino acid and its PTH derivatives.

Fast atom bombardment mass spectrometry (JEOL JMS-HX110/DA-5000 data system) of CGS gave a nominal M_r of 3618 which indicated that the carboxyl terminus of CGS is free. Since the carboxyl termini of all conotoxins characterized so far are amidated (Olivera et al., 1985), CGS is the first peptide toxin from *C. geographus* which has a free carboxyl terminus. The data also indicated that all cysteines are present as disulfides.

The peptide has 34 amino acids in a single polypeptide chain with a M_r of 3618 and has a net charge of +2.5 at neutral pH (1 Gla, 1 Asp, 1 Lys, 4 Arg, and 1 His residues, Figure 3).

Structural Properties of CGS. The main features of the amino acid sequence of CGS are as follows:

(i) CGS has a high content of cysteine and arginine, 6 Cys and 4 Arg in 34 residues. To confirm that the cysteine residues are present as disulfides, the intact peptide was S-carbamoylmethylated in the presence or absence of a reducing reagent, DTT. Six residues of CamCys were detected only in the presence of the reducing reagent, indicating that the six cysteine residues are present as disulfides. The cysteine residues of all other conotoxins with established amino acid

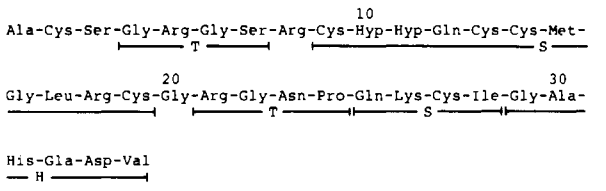

FIGURE 3: Amino acid sequence and secondary structure prediction for conotoxin GS from *C. geographus*. In the predicted secondary structure, regions are shown in α-helical (H), β-sheet (S), and β-turn (T) conformational states.

sequences are also known to be present as disulfides (Olivera et al., 1985). There are also many disulfide bonds in the neurotoxins of other venomous animals such as snakes, scorpions, and sea anemones. The high content of Arg gives CGS a high positive charge density, which is also a feature of several other conotoxins, such as μ-conotoxins. Therefore, CGS is a basic and tightly folded molecule, like most other peptide neurotoxins.

(ii) The most interesting features of the peptide toxin CGS are two posttranslational modifications in its sequence: hydroxylation of the proline residues at positions 10 and 11 and 4-carboxylation of the glutamic acid residue at position 32. There are two residues of Hyp, which are present in different sequences from those found in collagen, but like those of μ-conotoxins (Sato et al., 1983; Cruz et al., 1985) and ω-conotoxins (Olivera et al., 1984). The sequence Arg_8-Cys-Hyp-Hyp_{11} in CGS is different from the Gly-X-Y triplet characteristic of vertebrate collagen (Eyre, 1980). This may be due to a difference in hydroxylation enzymes. Hydroxylation occurs at two Pro residues in the NH_2-terminal half of CGS but not at position 24, so possibly Pro-24 is not recognized by the hydroxylation enzymes. μ-Conotoxins are a class of conotoxins with peptide chains of 22 amino acid residues containing 3 Hyp at positions 6, 7, and 17. Variants with Pro-6 and Pro-7 have been found, but none with Pro-17 (Cruz et al., 1985). One explanation of this difference is that the activity of the enzyme may be less efficient on the -Pro-Pro- sequence of μ-conotoxins. In contrast, in CGS, hydroxylation of the Pro_{10}-Pro_{11}- sequence is very efficient, but not that of the solitary Pro-24. Presumably the modification of proline residues in conotoxins reflects their effectiveness as substrates for hydroxylation and the specificity of the prolylhydroxylase. Another interesting factor is that CGS contains 4-carboxyglutamic acid in its primary structure. Gla is formed by posttranslational, vitamin K dependent carboxylation of specific glutamic acid residues in the polypeptide chain. The biological function of this unusual amino acid is best understood in blood coagulation proteins (Stenflo & Suttie, 1977; Nelsestuen, 1984) and bone osteocalcin (Gundberg et al., 1984). Gla has also been found in another conotoxin, called "sleeper" peptide (McIntosh et al., 1984). Intracerebral injection of this peptide induces a sleep-like state in mice. It seems likely that Gla in Gla-containing proteins binds Ca^{2+} and that this complex plays a role in the biological activity (Gundberg et al., 1984; Nelsestuen, 1984). We plan to study the function of Gla in CGS. As in the studies on prothrombin (Esmon et al., 1975; Bajaj et al., 1982) and osteocalcin (Poser & Price, 1979), it will be possible to deduce the function from the altered properties of non-4-carboxylated CGS. Various kinds of posttranslational modifications seem characteristic of biologically active peptides from *C. geographus* venom.

(iii) The structure of CGS was interpreted by computer methods for secondary-structure prediction (Chou & Fasman, 1978). We assumed that Hyp and Gla have similar probability parameters to Pro and Glu, respectively. As shown in Figure

Table V: Binding Constants of [^{3}H]Lys-TTX and [^{3}H]Pr-CGIIIA in Eel Electroplax Membranes and Rat Brain P_2 Fraction[a]

	B_{max} (pmol/mg)		K_d (nM)	
	[^{3}H]Lys-TTX	[^{3}H]Pr-CGIIIA	[^{3}H]Lys-TTX	[^{3}H]Pr-CGIIIA
eel electroplax[b]	10 ± 2	11 ± 2	1.4 ± 0.5	1.1 ± 0.2
rat brain	2.4 ± 0.1	*c*	0.9 ± 0.1	*c*

[a] Values were derived from Scatchard plots and are means ± SD of three to five determinations. [b] According to Yanagawa et al. (1987). [c] No saturable function.

3, CGS was predicted to have 18% α-helix, 44% β-sheet, and 24% β-turn structure.

(iv) The primary structures of CGS and other conotoxins are compared in Figure 4. Conotoxin GIIIA (A) is a Na channel inhibitor, while conotoxin MVIIA (B) is a Ca channel inhibitor (Olivera et al., 1987). The sequences of these toxins are arranged to obtain the maximum degree of homology. Although the pharmacological activity of CGS is very similar to that of CGIIIA, the homology in the sequences of CGS and CGIIIA is very limited except for the common region Pro_{24}-Gln-Lys-Cys_{27}. This region of the molecules may be important for the interactions between the receptor site on the Na channel and μ-conotoxins or CGS.

The N-terminal regions of CGS and conotoxin MVIIA, especially up to the Cys-9 residue, exhibit extensive homology, suggesting similarities in their binding sites to ion channels. Incidentally, it is interesting that these two channels have functional similarities, which suggest their common evolutionary origin (Hille, 1984).

The amino acid sequence of CGS was subjected to a computer-assisted search for homology with known sequences compiled in The Niigata University nucleic acid-protein sequence data-base system, but no significant homology has yet been found (Waterman et al., 1976).

[^{3}H]Pr-CGIIIA Is a Radioligand for Distinguishing between Neuronal and Muscle Na Channel Subtypes. Figure 5 compares the bindings of [^{3}H]Lys-TTX and [^{3}H]Pr-CGIIIA to the rat brain P_2 fraction. The specific [^{3}H]Lys-TTX binding, calculated as the difference between total and nonspecific bindings, was a saturable function of the [^{3}H]Lys-TTX concentration (Figure 5A).

Figure 5B shows the binding of [^{3}H]Pr-CGIIIA to the rat brain P_2 fraction under identical conditions to those in Figure 5A for the binding of [^{3}H]Lys-TTX. The total and nonspecific bindings were both linear, and the specific [^{3}H]Pr-CGIIIA binding was much less than the nonspecific binding. [^{3}H]-Lys-TTX binding reached 85–90% of the maximum at 5 nM, but there was no evidence for saturation of specific [^{3}H]Pr-CGIIIA binding at this concentration.

The electric organs of the electric eel are a specialized derivative of muscle (Luft, 1957) and have large quantities of Na channel at high density (Levinson et al., 1986). In eel

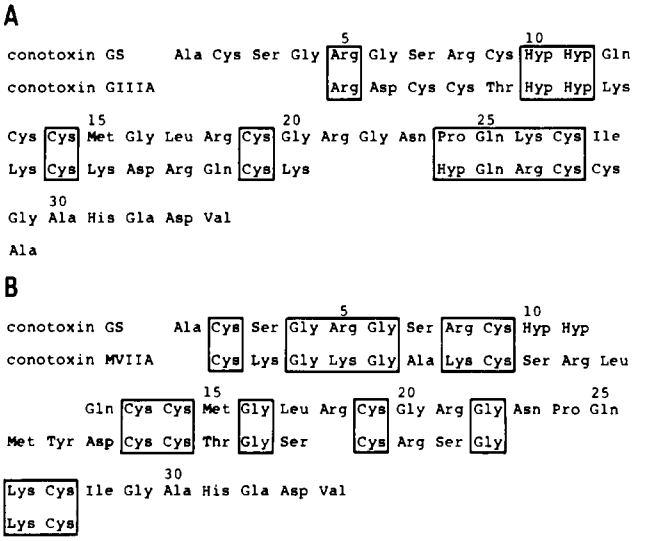

FIGURE 4: Comparison of the amino acid sequences of conotoxin GIIIA (A), conotoxin MVIIA (B), and conotoxin GS. Gaps are inserted to increase similarity. Identical residues and favored amino acid substitutions in two sequences are framed.

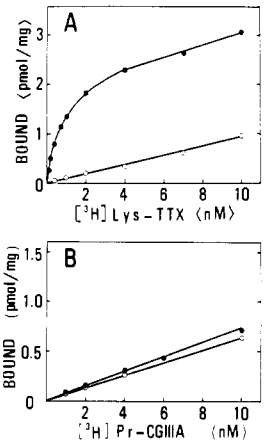

FIGURE 5: Binding of [^{3}H]Lys-TTX (A) and [^{3}H]Pr-CGIIIA (B) to the rat brain P_2 fraction. The rat brain P_2 fraction was incubated for 30 (A) or 60 min (B) at 4 °C with various concentrations of [^{3}H]Lys-TTX or [^{3}H]Pr-CGIIIA, and the bound radioactivity was measured by the centrifugation procedure. Total binding (●) and nonspecific binding (○), measured in the presence of 2 μM TTX (A) or 2 μM CGIIIA (B), are plotted versus the free [^{3}H]Lys-TTX (A) or [^{3}H]Pr-CGIIIA (B) concentration.

electroplax membranes, [^{3}H]Lys-TTX and [^{3}H]Pr-CGIIIA have similar affinities for the receptor site on Na channels, and the maximal binding capacity (B_{max}) values for the two are also almost the same (Table V). In addition, the two Na channel subtypes, referred to as muscle (eel electroplax) and nerve (rat brain) subtypes, have similar affinities for [^{3}H]-Lys-TTX. The Na channels of eel electroplax membranes

Table VI: Inhibition of [^{3}H]Lys-TTX and [^{3}H]Pr-CGIIIA Binding to Rat Brain P_2 Fraction, Skeletal Muscle Homogenates, and Eel Electroplax Membranes by Various Neurotoxins[a]

	inhibition of [^{3}H]Lys-TTX binding, IC_{50} (M)			inhibition of [^{3}H]Pr-CGIIIA binding, IC_{50} (M), to eel electroplax
toxin	rat brain	rat muscle	eel electroplax	
conotoxin GS	$>1 \times 10^{-5}$	8.8×10^{-7}	5.8×10^{-8}	4.8×10^{-8}
CGIIIA	2.9×10^{-6}	6.3×10^{-9}	1.0×10^{-9}	6.1×10^{-10} [b]
CGIIIB	1.1×10^{-6}	1.9×10^{-8}	1.2×10^{-9}	1.1×10^{-9} [b]
tetrodotoxin	2.3×10^{-9}	5.1×10^{-9}	4.5×10^{-9}	7.2×10^{-9} [b]
saxitoxin	9.2×10^{-10}	5.2×10^{-9}	6.0×10^{-9}	2.2×10^{-9} [b]

[a] In competition experiments, various concentrations of the indicated neurotoxins were incubated with eel electroplax membranes, rat skeletal muscle homogenates, or the rat brain P_2 fraction and 1 nM [^{3}H]Lys-TTX or 1 nM [^{3}H]Pr-CGIIIA. Data are means of three to five determinations. [b] According to Yanagawa et al. (1987).

have high affinity for [^{3}H]Pr-CGIIIA; but in the case of the Na channels of rat brain, neither the saturation nor the K_d value for specific binding of [^{3}H]Pr-CGIIIA can be accurately assessed.

These results indicate that [^{3}H]Pr-CGIIIA will be a useful probe for studying the number, density, distribution, and molecular properties of muscle Na channel subtypes.

Pharmacological Action of CGS. To assess the pharmacological action of the novel conotoxin, we examined its ability to displace the specific bindings of [^{3}H]Lys-TTX and [^{3}H]-Pr-CGIIIA to Na channels in various tissues in comparison with those of guanidinium toxins and μ-conotoxins (Table VI). The guanidinium toxins (TTX and STX) fully prevented the specific binding of [^{3}H]Lys-TTX with nearly the same affinity for the Na channels of rat brain and skeletal muscle and eel electroplax (IC_{50} values in the order of 10^{-9} M). μ-Conotoxins (CGIIIA and CGIIIB) inhibited the specific [^{3}H]Lys-TTX binding to rat skeletal muscle and eel electroplax Na channels with IC_{50} values of 10^{-9} to ca. 10^{-8} M order. However, μ-conotoxins have 1000-fold lower affinity (IC_{50} values in the order of 10^{-6} M) than TTX and STX for rat brain Na channels, as measured by competition binding experiments.

CGS inhibited specific [^{3}H]Lys-TTX binding to eel electroplax membranes and rat skeletal muscle homogenates in a concentration-dependent manner with IC_{50} values of 58 nM and 880 nM, respectively. On the other hand, it had only a slight inhibitory effect even at 10 μM on the binding of [^{3}H]Lys-TTX to the rat brain P_2 fraction. CGS is an effective competitor for TTX binding in electroplax and skeletal muscle but not in brain. These results indicate that the action of CGS is tissue specific, as is the case for μ-conotoxins, although the Na channels from three different sources are more sensitive to μ-conotoxins than to CGS.

CGS inhibited specific [^{3}H]Pr-CGIIIA binding to eel electroplax membranes in a concentration-dependent manner with an IC_{50} value of 48 nM. This corresponds to a K_i value of 22 nM, a value that is consistent with the K_i value (34 nM) obtained in competition binding with [^{3}H]Lys-TTX. These results support our previous conclusions that μ-conotoxins share a common binding site with TTX and STX on eel electroplax Na channels (Yanagawa et al., 1987). Competitive binding studies between μ-conotoxins and different Na channel subtypes indicate that the structures of the binding sites for TTX and STX in neuronal and muscle Na channel subtypes are heterogeneous, although considerable sequence homology exists between rat brain and eel electroplax Na channels (Noda et al., 1984, 1986). Thus CGS, like μ-conotoxins, can be used as a specific probe for distinguishing between neuronal and muscle Na channel subtypes. However, it is of interest that these two classes of peptide toxins, CGS and μ-conotoxins, share only limited amino acid sequence homology despite their pharmacological similarities.

In this work we demonstrated that CGS is a peptide toxin, containing the unusual amino acids Hyp and Gla, and that it can compete with [^{3}H]Lys-TTX and [^{3}H]Pr-CGIIIA for specific binding to eel electroplax membranes. Further investigations such as electrophysiological studies and three-dimensional structural analyses are required to elucidate the mechanisms of action of CGS in comparison with those of other well-known Na channel inhibitors.

ACKNOWLEDGMENTS

We thank Dr. Y. Kishi for a generous gift of saxitoxin. We are very grateful to Drs. A. Warashina and T. Ogura for their electrophysiological observations and to K. Tanaka for his FAB mass spectrometry analysis. We also thank S. Yamada for technical help, N. Takahashi for secretarial assistance, E. Ichihara and Dr. M. M. Dooley for critical reading of the manuscript, and Drs. T. Ono and T. Sato for encouragement.

REFERENCES

Bajaj, S. P., Price, P. A., & Russell, W. A. (1982) *J. Biol. Chem. 257*, 3726–3731.

Barchi, R. L. (1987) *Trends Neurosci. (Pers. Ed.) 10*, 221–223.

Catterall, W. A. (1980) *Annu. Rev. Pharmacol. Toxicol. 20*, 15–43.

Catterall, W. A. (1985) *Trends Neurosci. (Pers. Ed.) 8*, 39–41.

Catterall, W. A. (1986) *Annu. Rev. Biochem. 55*, 953–985.

Chicheportiche, R., Balerna, M., Lombet, A., Romey, G., & Ladzunski, M. (1980) *Eur. J. Biochem. 104*, 617–625.

Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251–276.

Cruz, L. J., Gray, W. R., Olivera, B. M., Zeikus, R. D., Kerr, L., Yoshikami, D., & Moczydlowski, E. (1985) *J. Biol. Chem. 260*, 9280–9288.

Esmon, C. T., Suttie, J. W., & Jackson, C. M. (1975) *J. Biol. Chem. 250*, 4095–4099.

Eyre, D. R. (1980) *Science (Washington, D.C.) 207*, 1315–1322.

Gonoi, T., Sherman, S. J., & Catterall, W. A. (1985) *J. Neurosci. 5*, 2559–2564.

Gonoi, T., Ohizumi, Y., Nakamura, H., Kobayashi, J., & Catterall, W. A. (1987) *J. Neurosci. 7*, 1728–1731.

Gray, W. R., Luque, A., Olivera, B. M., Barrett, J., & Cruz, L. J. (1981) *J. Biol. Chem. 256*, 4734–4740.

Gundberg, C. M., Hauschka, P. V., Lian, J. B., & Gallop, P. M. (1984) *Methods Enzymol. 107*, 516–544.

Hauschka, P. V., Carr, S. A., & Biemann, K. (1982) *Biochemistry 21*, 638–642.

Hermodson, M. A., Ericsson, L. H., Neurath, H., & Walsh, K. A. (1973) *Biochemistry 12*, 3146–3153.

Hille, B. (1984) *Ionic Channels of Excitable Membranes*, pp 371–383, Sinauer Associates, Sunderland, MA.

Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 197–199.

Hollenberg, M. D. (1985) in *Neurotransmitter Receptor Binding*, 2nd ed. (Yamamura, H. I., Enna, S. J., & Kuhar, M. J., Eds.) pp 1–39, Raven, New York.

Kobayashi, M., Wu, C. H., Yoshii, M. Narahashi, T., Nakamura, H., Kobayashi, J., & Ohizumi, Y. (1986) *Pfleugers Arch. 407*, 241–243.

Levinson, S. R., Duch, D. S., Urban, B. W., & Recio-Pinto, E. (1986) *Ann. N.Y. Acad. Sci. 479*, 162–178.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Luft, J. H. (1957) *J. Morphol. 100*, 113–129.

McIntosh, J. M., Olivera, B. M., Cruz, L. J., & Gray, W. R. (1984) *J. Biol. Chem. 259*, 14343–14346.

Moczydlowski, E., Olivera, B. M., Gray, W. R., & Stricharz, G. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5321–5325.

Narahashi, T. (1974) *Physiol. Rev. 54*, 813–889.

Nelsestuen, G. L. (1984) *Methods Enzymol. 107*, 507–516.

Noda, M., Shimazu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Matsuo, H., Raftery, M. A., Hirose, T., Inayama, S., Hayashida, H., Miyata, T., & Numa, S. (1984) *Nature (London) 312*, 121–127.

Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H., & Numa, S. (1986) *Nature (London) 320*, 188–192.

Ohizumi, Y., Nakamura, H., Kobayashi, J., & Catterall, W. A. (1986) *J. Biol. Chem. 261*, 6149–6152.

Olivera, B. M., McIntosh, J. M., Cruz, L. J., Luque, F. A., & Gray, W. R. (1984) *Biochemistry 23*, 5087-5090.
Olivera, B. M., Gray, W. R., Zeikus, R., McIntosh, J. M., Varga, J., Rivier, J., de Santos, V., & Cruz, L. J. (1985) *Science (Washington, D.C.) 230*, 1338-1343.
Olivera, B. M., Cruz, L. J., de Santos, V., LeCheminant, G. W., Griffin, D., Zeikus, R., McIntosh, J. M., Galyean, R., Varga, J., Gray, W. R., & Rivier, J. (1987) *Biochemistry 26*, 2086-2090.
Pappone, P. A. (1980) *J. Physiol. (London) 306*, 377-410.
Poser, J. W., & Price, P. A. (1979) *J. Biol. Chem. 254*, 431-436.
Price, P. A. (1983) *Methods Enzymol. 91*, 13-17.
Sato, S., Nakamura, H., Ohizumi, Y., Kobayashi, J., & Hirata, Y. (1983) *FEBS Lett. 155*, 277-280.
Stenflo, J., & Suttie, J. W. (1977) *Annu. Rev. Biochem. 46*, 157-172.
Ui, N. (1979) *Anal. Biochem. 97*, 65-71.
Waterman, M. S., Smith, T. F., & Beyer, W. A. (1976) *Adv. Math. 20*, 367-387.
Weiss, R. E., & Horn, R. (1986) *Science (Washington, D.C.) 233*, 361-364.
Yanagawa, Y., Abe, T., & Satake, M. (1986) *Neurosci. Lett. 64*, 7-12.
Yanagawa, Y., Abe, T., & Satake, M. (1987) *J. Neurosci. 7*, 1498-1502.

Human Rhinovirus 3C Protease: Cloning and Expression of an Active Form in *Escherichia coli*

Randell T. Libby,*,‡ David Cosman,‡ Marion K. Cooney,§ Janet E. Merriam,‖ Carl J. March,‖ and Thomas P. Hopp‖

Departments of Molecular Biology and Protein Chemistry, The Immunex Corporation, 51 University Street, Seattle, Washington 98101, and Department of Pathobiology SC-38, University of Washington, Seattle, Washington 98195

Received February 9, 1988; Revised Manuscript Received April 6, 1988

ABSTRACT: A cDNA encoding the viral protease from the 3C region of human rhinovirus type 14 was expressed in *Escherichia coli* through the use of a periplasmic secretion vector. The recombinant protease contained an eight amino acid N-terminal extension that enabled its detection by a specific antibody. It was expressed at a level of approximately 1 mg/L of *E. coli* culture. Biological activity of the protease was assessed in vitro by using a chemically synthesized peptide consisting of a consensus picornavirus protease cleavage site, Arg-Ala-Glu-Leu-Gln-Gly-Pro-Tyr-Asp-Glu. The peptide was cleaved by the recombinant protease at the Gln-Gly bond, generating the product peptides Arg-Ala-Glu-Leu-Gln and Gly-Pro-Tyr-Asp-Glu, which could be separated from the substrate peptide by reversed-phase HPLC. An in vitro assay for the rhinovirus 3C protease was developed by observing the rate of disappearance of the substrate peak from chromatograms of the supernatants of digestion mixtures.

Human rhinoviruses (HRVs) belong to one genus of the family picornaviridae and are the leading cause of the common cold in man (Stott et al., 1972; Gwaltney, 1975). Other members of the picornavirus family include the entero (polio, echo, coxsachie, hepatitis A), aphtho- [foot and mouth disease (FMDV)], and cardioviruses [mengo, encephalomyocarditis (EMCV)] (Cooper et al., 1978). These latter groups have much in common with the rhinoviruses including their occurrence as icosehedral virions containing a single-stranded RNA genome. The antigenic features on the surfaces of picornaviruses are complex, and among HRVs, 100 serotypes have been identified (Hamparian et al., 1987). The high incidence of the common cold may be explained, at least in part, by this antigenic variability and the cocirculation within the community of several serotypes simultaneously (Gwaltney, 1975). Hence, conventional vaccination programs are virtually useless against the rhinoviruses, making a search for alternative methods for inactivation of these viruses essential.

Controlled proteolysis appears to be crucial to the life cycle of the rhinoviruses, as is evident from the fact that at least two, and probably three, proteolytic enzymes are encoded in the viral genome (Arnold et al., 1987). Like other picornaviruses, the rhinovirus contains a single, positive stranded RNA genome of about 7500 nucleotides (Rossman et al., 1985; Medappa et al., 1971; Dimmock, 1966; Brown et al., 1970; Stanway et al., 1984; Callahan et al., 1985) that encodes a single long open reading frame that is translated into a large precursor protein of more than 200 000 daltons. This polyprotein is processed into the four virion polypeptides VP1, VP2, VP3, and VP4 as well as a number of noncapsid proteins by cleavages made by the viral proteases.

This report deals with the cloning and expression of the cDNA that encodes the rhinovirus 3C protease. A novel expression vector was developed that places an eight amino acid immunoreactive "peptide" extension N-terminal to the 3C protease gene. The peptide extension was found to be useful for partial purification of the recombinant protease. Moreover, we have developed a synthetic substrate, based on the predicted HRV-14 polyprotein cleavage sites (Callahan et al., 1985), for analysis of the recombinant protease activity.

* Author to whom correspondence should be addressed at his present address: Department of Genetics, SK-50, University of Washington, Seattle, WA 98195.
‡ Department of Molecular Biology, The Immunex Corp.
§ University of Washington.
‖ Department of Protein Chemistry, The Immunex Corp.

0006-2960/88/0427-6262$01.50/0