Structure–Function Relationships and Site of Action of Apamin, a Neurotoxic Polypeptide of Bee Venom with an Action on the Central Nervous System†

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ABSTRACT: Specific chemical modifications of apamin have been used to study the residues involved in its toxic action. Transformation of Lys_4 into homoarginine did not affect toxicity. Modification of the α-amino group of Cys_1 and of the ϵ-amino group of Lys_4 by acetic anhydride or fluorescamine decreased toxicity only by a factor of 2.5–2.8. Modification of the γ-carboxylate of Glu_7 with glycine ethyl ester in the presence of a soluble carbodiimide decreased toxicity by a factor of 2. Diethyl pyrocarbonate treatment of the imidazole side chain of His_{18} decreased toxicity by a factor of 2.6. Thus none of these residues is essential for toxicity. However, combined modification of amino groups and of the imidazole side chain of His_{18} completely abolished biological activity. Complete loss of toxicity also resulted from reduction and alkylation of both disulfide bridges, from chemical modification with cyclohexanedione of Arg_{13} and Arg_{14}, and from removal of Arg_{14} of acetylated apamin by digestion with trypsin. Incorporation of radioactive acetyl groups on both amino groups of apamin gave an active labeled toxin which has been used to localize the site of action of apamin in the spinal cord, principally in the lumbar part of the neuraxis.

The structure and properties of apamin from bee venom have been studied by Habermann and his group (1972). Apamin (Figure 1) is a polypeptide of 18 amino acids with two disulfide bridges (Shipolini et al., 1967; Haux et al., 1967; Callewaert et al., 1968) and is the smallest known venom neurotoxin.

Apamin is the only polypeptide neurotoxin, as far as we know, that passes the blood-brain barrier. It shows excitatory neurotoxic effects on the central nervous system. Intravenous injection of lethal or sublethal doses of apamin in mice causes extreme uncoordinated hypermotility. A lethal dose induces tonic convulsions followed by respiratory distress and death (Habermann, 1972).

Apamin differs from other venom toxins such as snake neurotoxins; these are peptides of 61–74 amino acids cross-linked by four or five disulfide bridges and produce a curarizing action by blockade of the acetylcholine receptor at the neuromuscular junction (see, for example, Lee (1970)). It also differs from scorpion neurotoxins, which have 63–64 amino acids cross-linked by four disulfide bridges and which act on the excitable membranes of axons (Adam et al., 1966; Koppenhöfer and Schmidt, 1968; Narahashi et al., 1972; Fayet et al., 1974).

This work had two aims: first to identify the amino acid residues which are particularly important for the toxic function and second, to incorporate selectively a radioactive label which would permit direct determination of the nature of the target tissue of the toxin in the nervous system.

Materials and Methods

Materials. The work described in this paper has been carried out with 600 mg of apamin which was extracted from bee venom by the purification technique described by Habermann and Reiz (1964, 1965a,b) adapted for large scale purification.

Strychnine (Merck) was labeled by catalytic tritium exchange at the Commissariat à l'Energie Atomique (France). The tritiated strychnine was purified from degradation products as described by Young and Snyder (1973). The specific radioactivity of [^{3}H]strychnine was 0.26 Ci/mmol.

[^{3}H]- and [^{14}C]acetic anhydride were obtained from the Commissariat à l'Energie Atomique (France).

Fluorescamine (Fluram) was obtained from Roche (Neuilly sur Seine, France).

Preparation of Apamin Derivatives. Acetylation. Amino groups of apamin were acetylated with [^{14}C]- or [^{3}H]acetic anhydride under the conditions described by Chauvet and Acher (1967) for the pancreatic trypsin inhibitor. The excess of reagent was eliminated by filtration on Sephadex G-25 and the number of acetyl groups incorporated per mole of apamin was estimated from radioactivity measurements in a Packard TriCarb scintillation spectrometer, Model 3375.

Fluorescamine Treatment. Fluorescamine (4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione) reacts with primary amines to form intensely fluorescent substances (Weigele et al., 1972; Udenfriend et al., 1972). The reaction between apamin and fluorescamine was carried out as follows: apamin (final concentration 0.4 m*M*) was dissolved in 2.5 ml of 50 m*M* sodium phosphate buffer (pH 7.5) containing 0.15 *M* NaCl; 0.5 ml of 10 m*M* fluorescamine in acetone was rapidly added to the apamin with stirring at room temperature. The modification reaction was complete within 1 min. The number of amino groups modified by fluorescamine was estimated by back titration with [^{14}C]acetic anhydride.

Guanidination. The ϵ-amino group of Lys_4 was transformed into homoarginine by reacting apamin with *O*-methylisourea hydrogen sulfate as described by Chauvet

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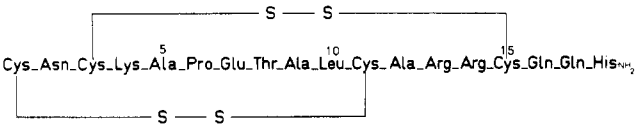

FIGURE 1: The sequence of apamin (Shipolini et al., 1967; Haux et al., 1967; Callewaert et al., 1968).

and Acher (1967) for the pancreatic trypsin inhibitor. Apamin (4 mg/ml) was incubated at pH 11.0, 4°, with *O*-methylisourea hydrogen sulfate (0.5 *M*). The reaction was stopped after 6 days by lowering the pH to 3.0 with 5 *N* HCl. The modified apamin was separated from small molecules by Sephadex G-25 filtration in 1 m*M* HCl. The extent of guanidination was evaluated by amino acid analysis and by back titration with [^{14}C]acetic anhydride.

Modification of the Carboxylate Side Chain. The carboxylic function of Glu_7 was modified by reaction with [^{14}C]glycine ethyl ester and 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide using the method of Hoare and Koshland (1967). The reaction was carried out for 24 hr at pH 4.0, 25°, in 1 *M* glycine ethyl ester and 62.5 m*M* 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide; the apamin concentration was 3 mg/ml. The excess of reagent was eliminated by filtration through a Sephadex G-25 column equilibrated with 1 m*M* HCl. The amount of glycine ethyl ester incorporated was estimated from radioactivity measurements and amino acid analysis.

Modification of Histidine. (1) CARBETHOXYLATION. The imidazole function of His_{18} was carbethoxylated with diethyl pyrocarbonate under conditions described by Grousselle et al. (1973) for yeast hexokinase. Apamin (final concentration 0.2–0.5 m*M*) was incubated at 0°, pH 7.5, in 50 m*M* phosphate buffer containing 0.15 *M* NaCl. Carbethoxylation was initiated by adding an aliquot of a freshly prepared solution of diethyl pyrocarbonate in ethanol (final concentration 0.5–5 m*M*). The modification was followed by recording the increase in absorbance at 242 nm in a Varian Tectron Model 625 spectrophotometer equipped with an A-25 recorder and a thermostated cell holder. The molar extinction coefficient is 2900 cm^{-1} M^{-1} at pH 7.5 (Tudball et al., 1972). Back titration with [^{14}C]acetic anhydride was used to determine if free amino groups had reacted.

(2) DECARBETHOXYLATION. Carbethoxyapamin was incubated at pH 7.5 in a 50 m*M* phosphate buffer containing 0.15 *M* NaCl either at 0° in the presence of 0.3 *M* hydroxylamine or at 37° without hydroxylamine. The kinetics of decarbethoxylation were followed by the decrease in absorbance at 242 nm.

Modification of Arginine. The guanidine side chain of arginines 13 and 14 were modified by 1,2-cyclohexanedione according to the method of Toi et al. (1967). Apamin (final concentration 3 m*M*) was incubated in the dark at room temperature in 5 ml of 0.2 *N* NaOH containing 45 mg of 1,2-cyclohexanedione. The reaction was stopped after 2 hr by lowering the pH to 2.7 with 5 *N* HCl. The product was filtered through a Millipore filter, then desalted in a Sephadex G-25 column equilibrated with 1 m*M* HCl and lyophilized. The number of modified arginine residues and the integrity of other amino acid side chains were determined by amino acid analysis with a Beckman Model 120C automatic amino acid analyzer.

Modification of Disulfide Bridges. Both disulfide bridges of apamin can be reduced by sodium borohydride. Apamin (0.5 m*M*) was incubated at 20°, pH 9.6, under nitrogen, in a 0.1 *M* sodium borohydride solution. Aliquots were taken at different times and titrated to pH 2.0 with 1 *N* HCl to destroy excess borohydride. After 15 min the newly formed SH groups were titrated by the Ellman reagent (Ellman, 1959). The fully reduced apamin solution was alkylated with [^{14}C]iodoacetamide to give a carboxamidomethylated derivative as previously described (Vincent et al. 1971).

Reoxidation of the fully reduced apamin was also followed with the Ellman technique at pH 8.5, 25°.

Trypsin-Catalyzed Hydrolysis of Acetylated Apamin. Apamin was acetylated before trypsin treatment to protect the neurotoxin against cleavage of the Lys_4–Ala_5 bond. Acetylated apamin (26 mg) was dissolved in 5 ml of 20 m*M* Tris buffer (pH 8.0) containing 0.15 *M* NaCl and 10 m*M* $CaCl_2$. Trypsin (1.2 mg, TPCK-treated, devoid of chymotryptic activity) was added and the mixture was incubated at 25° for 24 hr. Trypsin was then separated from hydrolyzed apamin by filtration of the incubation mixture on a Sephadex G-25 column (2 × 54 cm) equilibrated with 1 m*M* HCl. Fractions corresponding to the enzyme and the cleaved apamin were pooled, lyophilized, and identified by amino acid analysis.

LD_{50} Measurements. Toxicity was determined as LD_{50} according to Miranda et al. (1970) by subcutaneous injection of native or modified apamin into Swiss mice of 20 ± 3 g. LD_{50} values for native apamin have been systematically redetermined for all new lots of mice. Depending upon the lot, the LD_{50} for native apamin varied between 60 and 80 μg/20 g of body weight.

Titration of Apamin. Desalted apamin (48 μmol) was dissolved in 2 ml of a solution containing 0.5 *M* NaCl and 10 m*M* HCl. Titration with 2 *N* NaOH was carried out at 25° under nitrogen in a Radiometer pH-Stat TTT 11 equipped with an SBR_2C recorder.

Localization of Target Tissues for Strychnine and Apamin in Mice. Mice weighing 30 ± 3 g were killed by subcutaneous injection of supralethal doses (about three times higher than the LD_{50} value) of either ^{14}C-acetylated apamin (0.25 Ci/mol) or [^{3}H]strychnine (2 Ci/mol). Immediately after death the organs were carefully removed by dissection and homogenized in H_2O with a Teflon–glass homogenizer (Type A, Thomas, Philadelphia). The incorporation of toxin into each organ was monitored by radioactivity measurements of the homogenates.

Results

Titration of Apamin. Apamin is a small peptide of 18 amino acids (Figure 1). Four of the amino acid side chains are ionizable. The experimental titration curve of apamin is perfectly well described by a calculated curve for the titration of four groups with p*K* values of 4.1, 6.0, 7.3, and 10.6 at 25°. These values have been assigned to the γ-carboxylate of Glu_7, the imidazole of His_{18}, the α-NH_2 of Cys_1, and the ε-NH_2 of Lys_4, respectively. All these functions have normal p*K*'s.

Chemical Modification of Amino Groups. The ε-amino group of Lys_4 is selectively guanidinated with *O*-methylisourea hydrogen sulfate; other amino acids remain intact. Selective transformation of Lys_4 into homoArg does not affect the toxicity of apamin (Table I).

Acetylation with [^{14}C]acetic anhydride occurs on the α-amino group of Cys_1 and the ε-amino group of Lys_4. Diacetylation of apamin decreases the toxicity by a factor of 2.5 (Table I).

Table I: Importance of Ionizable Functions for the Toxicity of Apamin.

Modified Functions	Modification	Experimental Evidence for the Extent of the Modification	LD_{50} (modified) / LD_{50} (native)
ϵ-Amino of Lys_4	Guanidination	Amino acid analysis (disappearance of lysine)	1.0
ϵ-Amino of Lys_4 α-Amino of Cys_1	Acetylation	Incorporation of 1.9 [^{14}C]acetyl group	2.5
ϵ-Amino of Lys_4 α-Amino of Cys_1	Fluorescamine treatment	Back titration with [^{14}C]acetic anhydride (no incorporation)	2.8
γ-Carboxyl of Glu_7	Formation of an amide bond with glycine ethyl ester	Incorporation of 1.0 [^{14}C]glycine ethyl ester; appearance of 1 glycine residue as shown by amino acid analysis	2.0
Imidazole of His_{18}	Carbethoxylation	Formation of 1 carbethoxyhistidine from ϵ_{242nm}	2.6
Carbethoxyhistidine[a]	Decarbethoxylation	Disappearance of 1 carbethoxyhistidine from ϵ_{242nm}	1.0
Imidazole of His_{18} ϵ-amino of Lys_4 α-amino of Cys_1	Carbethoxylation and acetylation	Formation of 1 carbethoxyhistidine from ϵ_{242nm} and incorporation of 1.9 [^{14}C]acetyl groups	>10[b]
Guanidine functions of Arg_{13} and Arg_{14}	1,2-Cyclohexanedione treatment	Amino acid analysis (disappearance of arginines)	>10[b,c]
Arg_{14} of Cys_1, Lys_4 diacetylapamin	Cleavage by trypsin	Amino acid analysis (disappearance of 1 Arg)	>10[b]

[a] Apamin with 1.0 carbethoxyhistidine group was decarbethoxylated by treatment with 0.3 *M* hydroxylamine at 0°, pH 7.5 for 2 hr. [b] Doses of the modified derivative of apamin corresponding to tenfold the LD_{50} value of the unmodified apamin neither killed mice nor produced any characteristic symptom of apamin intoxication. [c] Cyclohexanedione treatment involves the use of very drastic experimental conditions (0.2 *N* NaOH, 2 hr, 25°). In a control experiment, apamin was incubated under these conditions but in the absence of cyclohexanedione. The resulting apamin has a toxicity three times lower than that of native apamin. The value taken in the table (LD_{50} (modified)/LD_{50} (native)) is in fact the ratio between the LD_{50} value obtained after cyclohexanedione treatment and the LD_{50} value obtained after the control incubation at alkaline pH.

Fluorescamine is a reagent with a high specificity for amino groups (Weigele et al., 1972; Udenfriend et al., 1972). Treatment of apamin by fluorescamine blocks both the α-amino group of Cys_1 and the ϵ-amino group of Lys_4 as demonstrated by the fact that the fluorescamine-modified derivative of apamin cannot incorporate radioactive acetyl groups from [^{14}C]acetic anhydride. The toxicity of apamin is decreased by a factor of 2.8 after modification by fluorescamine (Table I).

All these data taken together clearly indicate that neither the α-amino group of Cys_1 nor the ϵ-amino group of Lys_4 is essential for the neurotoxicity of apamin.

Chemical Modification of the Carboxylate Side Chain of Glu_7. The selective chemical modification of the sole carboxylate of the apamin sequence, the side chain of Glu_7 (the C-terminal residue is His_{18}-amide), has been carried out with glycine ethyl ester after activation of the carboxyl by a water-soluble carbodiimide (Hoare and Koshland, 1967) (Table I). The formation of an amide bond between the γ-carboxylic function of Glu_7 and the glycine ester decreases the toxicity of apamin only by a factor of 2 (Table I). Glu_7 does not appear to be essential for the neurotoxicity of apamin.

Chemical Modification of the Side Chain of His_{18}. Diethyl pyrocarbonate is one of the best reagents for the imidazole ring of histidine (Mühlrad et al., 1967; Ovadi et al., 1967; Grousselle et al., 1973). Apamin possesses a single histidine residue, His_{18}. The carbethoxylation of this histidine residue by diethyl pyrocarbonate is presented in Figure 2A. Kinetics of the reaction are pseudo-first-order and the imidazole ring is completely carbethoxylated under the experimental conditions. Carbethoxylated apamin still incorporates 1.9 [^{14}C]acetyl groups/mol of toxin when treated with [^{14}C]acetic anhydride. The α-amino and the ϵ-amino groups of carbethoxylated apamin also freely react with fluorescamine to give an apamin derivative with the same fluorescence spectrum as that of apamin directly treated with fluorescamine. Thus at pH 7.5 and 0° diethyl pyrocarbonate modifies neither the α-amino group of Cys_1 nor the ϵ-amino group of Lys_4. A selective modification of the histidine residues of yeast hexokinase with diethyl pyrocarbonate has also been observed at 0°, pH 7.5, by Grousselle et al. (1973).

Carbethoxylation of the imidazole side chains with diethyl pyrocarbonate is a reversible process (Mühlrad et al., 1967; Melchior and Farney, 1970; Grousselle et al., 1973). Figure 2B shows that the carbethoxylation of apamin is completely reversed in 0.3 *M* hydroxylamine at 0°, pH 7.5, according to a first-order process. First-order and complete decarbethoxylation can also occur without hydroxylamine at 37°, pH 7.5. The half-life of the carbethoxylated apamin derivative is more than 20 hr (Figure 2C).

Carbethoxylation of the imidazole side chain of His_{18} decreases the toxicity by a factor of 2.6 (Table I).

Combined modification of the imidazole side chain of His_{18} by diethyl pyrocarbonate and of the α-amino and ϵ-amino groups of Cys_1 and Lys_4, respectively, by [^{14}C]acetic anhydride drastically alters the toxicity (Table I). Al-

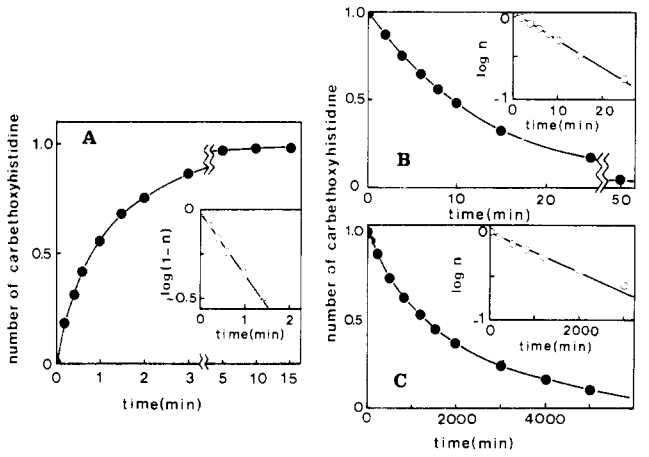

FIGURE 2: Reversible modification of the imidazole ring of His_{18} by diethyl pyrocarbonate. Carbethoxylation and decarbethoxylation were followed by spectrophotometric measurements at 242 nm with $\epsilon_{242\,nm}$ 2900 cm^{-1} M^{-1} for carbethoxyimidazole at pH 7.5. (A) Kinetics of carbethoxylation of apamin (0.13 mM) by diethyl pyrocarbonate (1.7 mM) at 0°, pH 7.5: (●) Time course of the reaction; (○) pseudo-first-order representation of the data. (B) Kinetics of decarbethoxylation of carbethoxyapamin (0.13 mM) by 0.3 M hydroxylamine at 0°, pH 7.5: (●) time-course of the reaction; (○) pseudo-first-order representation of the data. (C) Kinetics of spontaneous decarbethoxylation of carbethoxyapamin (0.13 mM) at 37°, pH 7.5: (○) time course of the reaction; (○) pseudo-first-order representation of the data. n is the number of carbethoxyhistidine group in apamin.

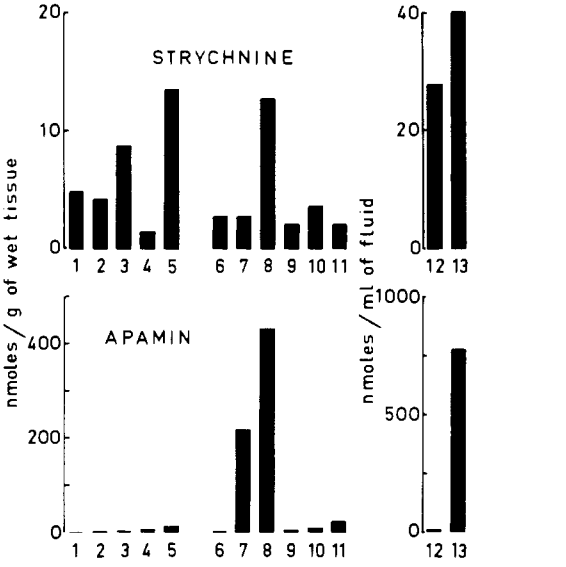

FIGURE 3: Comparison of the organ localization of strychnine and apamin in mice killed by supralethal doses of each toxin. Mice were killed by doses corresponding to threefold the LD_{50} value of either [^{3}H]strychnine or [^{14}C]acetylapamin. Organs were dissected immediately after death and homogenized in H_2O. The toxin uptake of each organ was estimated from radioactivity measurement of the homogenates. 1, liver; 2, heart; 3, lung; 4, testes; 5, kidney; 6, cervical spinal cord; 7, dorsal spinal cord; 8, lumbar spinal cord; 9, medulla oblongata; 10, cerebellum; 11, brain minus cerebellum; 12, blood; 13, urine.

though neither the imidazole of His_{18} nor the amino groups of Cys_1 and Lys_4 seem to be essential by themselves, modification of the three basic functions renders the toxin totally inactive.

Chemical Modification of Arg_{13} and Arg_{14}. The two arginines of apamin are contiguous in the C-terminal end of the sequence. Amino acid analysis of the apamin derivative obtained after cyclohexanedione treatment indicates a selective and complete modification of the two arginines. The extent of lysine modification is less than 10%. Total modification of Arg_{13} and Arg_{14} side chains results in complete loss of toxicity (Table I).

Reduction and Reoxidation of Disulfide Bridges. Reduction of both disulfide bridges of apamin by sodium borohydride followed by alkylation of the newly formed -SH groups by iodoacetamide completely eliminates the neurotoxicity of apamin. This is a confirmation of previous results obtained by Habermann (1972). Spontaneous reoxidation of the fully reduced apamin occurs readily at pH 8.5, 25°, in normal atmosphere. It is accompanied by a precipitation of a significant amount of the neurotoxin (30–40%). After elimination of the insoluble material by filtration on Millipore filters, LD_{50} measurements indicate that the toxicity of the soluble reoxidized apamin is decreased by a factor of 1.5 as compared to native apamin.

Trypsin Cleavage of Acetylated Apamin. The previous section has shown the essentiality of Arg_{13} and/or Arg_{14} for the biological activity of the toxin. Another way of studying the importance of the sequence of the two arginine residues is to attack it with trypsin. After acetylation of Lys_4 with acetic anhydride, it is no longer possible to cleave the Lys_4-Ala_5 bond with trypsin. The only possible cleavages are at the Arg_{13}-Arg_{14} and the Arg_{14}-Cys_{15} bonds. Amino acid analysis shows that trypsin cleavage of acetylated apamin containing intact disulfide bridges gives a derivative lacking one arginine residue, presumably Arg_{14}. Trypsin modification of acetylated apamin gives a two-chain toxin in which the two chains, Cys_1···Arg_{13} and Cys_{15}···His_{18}, are connected by disulfide bridge 3–15. This new derivative of apamin is completely devoid of toxic activity (Table I).

The Site of Action of Apamin is Located in the Spinal Cord. We have taken advantage of the fact that the amino groups of Cys_1 and Lys_4 can be acetylated with only a minor change in toxicity to prepare a radioactive diacetylated derivative of apamin using [^{14}C]acetic anhydride.

A supralethal dose of radioactive acetylated apamin is injected into a mouse. After death, the radioactive material is found predominantly in the spinal cord, mainly in the lumbar and dorsal parts of the spinal cord (Figure 3). Brain, kidney, cerebellum, medulla, testes, and blood contain small, but significant amounts of radioactivity; the other organs contain only traces.

Biochemical and neurophysiological evidence suggests that glycine is a major inhibitory neurotransmitter in the mammalian central nervous system. Glycine appears to act as a natural inhibitory transmitter in the spinal cord but not in the cerebral cortex. Strychnine antagonizes the hyperpolarizing actions of glycine at spinal synapses, where it also antagonizes naturally occurring synaptic inhibition (for references, see Young and Snyder 1973)). [^{3}H]Strychnine was found to bind selectively to synaptic membrane fractions of the spinal cord (Young and Snyder, 1973). We have compared in Figure 3 the localization of radioactive apamin in an animal killed by apamin and the localization of radioactive strychnine of an animal killed by an approximately equimolar amount of strychnine. LD_{50} values (expressed in nanomoles of toxin) for strychnine and apamin are very similar: 45 and 30 nmol of toxin/20 g of body weight, respectively. [^{3}H]Strychnine was found in the kidney, in the lungs, in the blood, in urine, and in the lumbar part of the spinal cord. In spite of the fact that strychnine is known to

act at the level of the spinal cord, significant amounts of the radioactive molecule are found in all tissues analyzed for their strychnine contents. The comparison in Figure 3 demonstrates clearly that apamin is more specific than strychnine for the spinal cord. Binding capacities of the lumbar part of the spinal cord for strychnine and apamin evaluated from Figure 3 are 13 and 430 nmol of toxin/g of wet tissue, respectively. This difference in the number of toxin binding sites suggests that receptors of the two toxic compounds are different.

Discussion

This work has shown that the α-amino group of Cys_1, the ϵ-amino group of Lys_4, the carboxylate side chain of Glu_7, and the imidazole group of His_{18} are not essential for the toxic activity of apamin. However, only guanidination of Lys_4 retains the high toxicity of the native neurotoxin. All other chemical modification decreases the toxic effect (measured as LD_{50}) by a factor of 2–3. A synergistic effect is observed when several functions are modified. It has been found for example that a neurotoxin in which the α- and ϵ-amino groups of Cys_1 and Lys_4 have been acetylated and the imidazole of His_{18} has been carbethoxylated is devoid of activity.

The most important part of the apamin sequence for neurotoxic activity appears to be the C-terminal region containing the two arginine residues. Chemical modification of Arg_{13} and Arg_{14} eliminates toxicity. Removal of Arg_{14} with formation of a two-chain toxin by trypsic cleavage of the acetylated derivative also destroys the activity.

An interesting application of this work is that it permits incorporation of either radioactive or fluorescent labels in a part of the toxin sequence that is not essential for activity, that is on the amino groups of Cys_1 and Lys_4. Injection of radioactive toxin has shown that its site of action is localized in the spinal cord. This conclusion is in agreement with neurophysiological studies which have shown that apamin affects spinal reflexes (Wellhöner, 1969). Apamin appears mainly to augment polysynaptic reflexes and to render excitatory polysynaptic pathways more effective than inhibitory polysynaptic mechanisms.

Work is now in progress to identify the receptor of apamin in membrane fractions and to understand the molecular mechanism of the toxic action of this polypeptide. The incorporation of a fluorescent label by treatment of the toxin with fluorescamine will be used in histochemical experiments.

Acknowledgments

The authors are very grateful to Professor F. Miranda and his group for interesting discussions, to Dr. A. Bourgois for amino acid analysis and helpful comments, and to Professor B. Kassel for very careful reading of the manuscript and interesting discussions.

References

Adam, K. R., Schmidt, H., Stampfli, R., and Weiss, C. (1966), *Br. J. Pharmacol. 26,* 666–672.

Callewaert, G. L., Shipolini, R., and Vernon, C. A. (1968), *FEBS Lett. 1,* 111–113.

Chauvet, J., and Acher, R. (1967), *Biochem. Biophys. Res. Commun. 27,* 230–235.

Corrado, A. P., Antonio, A., and Diniz, C. R. (1968), *J. Pharmacol. Exp. Ther. 164,* 253–258.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70–77.

Fayet, G., Couraud, F., Miranda, F., and Lissitzky, S. (1974), *Eur. J. Pharmacol. 27,* 165–174.

Grousselle, M., Thiam, A. A., and Pudles, J. (1973), *Eur. J. Biochem. 39,* 431–441.

Habermann, E. (1972), *Science 177,* 314–322.

Habermann, E., and Reiz, K. G. (1964), *Naturwissenschaften 51,* 61.

Habermann, E., and Reiz, K. G. (1965a), *Biochem. Z. 341,* 451–466.

Habermann, E., and Reiz, K. G. (1965b), *Biochem. Z. 343,* 192–203.

Haux, P., Sawerthai, H., and Habermann, E. (1967), *Hoppe-Seylers Z. Physiol. Chem. 348,* 737–743.

Hoare, D. G., and Koshland, D. E. (1967), *J. Biol. Chem. 242,* 2447–2453.

Koppenhöfer, E., and Schmidt, H. (1968), *Arch. Ges. Physiol. 303,* 133–138.

Lee, C. Y. (1970), *Clin. Toxicol. 3,* 457–472.

Melchior, W. B., Jr., and Farney, D. (1970), *Biochemistry 9,* 251–258.

Miranda, F., Kopeyan, C., Rochat, H., Rochat, C., and Lissitzky, S. (1970), *Eur. J. Biochem. 16,* 514–523.

Mühlrad, A., Hegyi, G., and Toth, G. (1967), *Acta Biochim. Biophys. Acad. Sci. Hung. 2,* 19–29.

Narahashi, T., Shapiro, B. I., Deguchi, T., Scuka, M., and Wang, C. M. (1972), *Am. J. Physiol. 222,* 850–857.

Ovadi, J., Libor, S., and Elödi, P. (1967), *Acta Biochim. Biophys. Acad. Sci. Hung. 2,* 455–458.

Shipolini, R., Bradbury, A. F., Callewaert, G. L., and Vernon, C. A. (1967), *Chem. Commun.,* 679–680.

Toi, K., Bynum, E., Norris, E., and Itano, H. A. (1967), *J. Biol. Chem. 242,* 1036–1043.

Tudball, N., Bailey-Wood, R., and Thomas, P. (1972), *Biochem. J. 129,* 419–425.

Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., and Weigele, M. (1972), *Science 178,* 871–872.

Vincent, J. P., Chicheportiche, R., and Lazdunski, M. (1971), *Eur. J. Biochem. 23,* 401–411.

Weigele, M., De Bernardo, S., Tengi, J., and Leimgruber, W. (1972), *J. Am. Chem. Soc. 94,* 5927–5930.

Wellhöner, H. H. (1969), *Nauyn-Schmiedeberg Arch. Pharmakol. Exp. Pathol. 262,* 29–41.

Young, A. B., and Snyder, S. H. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2832–2836.