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Scyllatoxin, a Blocker of Ca^{2+}-Activated K^+ Channels: Structure-Function Relationships and Brain Localization of the Binding Sites†

Patrick Auguste,‡ Michel Hugues,‡ Christiane Mourre,‡ Danielle Moinier,‡ Andrê Tartar,§ and Michel Lazdunski*,‡
Institut de Pharmacologie Molêculaire et Cellulaire, 660 Route des Lucioles, Sophia Antipolis, 06560 Valbonne, France, and Institut Pasteur de Lille, Service de Chimie des Molêcules, 1 Rue du Professeur Calmette, 59019 Lille, France
Received August 12, 1991; Revised Manuscript Received October 7, 1991

ABSTRACT: Chemical modifications of scyllatoxin (leiurustoxin I) have shown that two arginines in the sequence, Arg_6 and Arg_{13}, are essential both for binding to the Ca^{2+}-activated K^+ channel protein and for the functional effect of the toxin. His_{31} is important both for the binding activity of the toxin and for the induction of contractions on taenia coli. However, although its iodination drastically decreases the toxin activity, it does not abolish it. Chemical modification of lysine residues or of Glu_{27} does not significantly alter toxin binding, but it drastically decreases potency with respect to contraction of taenia coli. The same observation has been made after chemical modification of the lysine residues. The brain distribution of scyllatoxin binding sites has been analyzed by quantitative autoradiographic analysis. It indicates that apamin (a bee venom toxin) binding sites are colocalized with scyllatoxin binding sites. The results are consonant with the presence of apamin/scyllatoxin binding sites associated with Ca^{2+}-activated K^+ channels. High-affinity binding sites for apamin can be associated with very-high-affinity (<70 pM), high-affinity (~100-500 pM), or moderate-affinity (>800 pM) binding sites for scyllatoxin.

Polypeptide toxins are important tools for the study of ionic channels (Strong, 1990; Betz, 1990; Moczydlowski et al., 1988; Cook & Quast, 1990; Dreyer, 1990). The venom of the scorpion *Leiurus quinquestriatus hebraeus* contains two different types of polypeptide toxins capable of blocking two different types of Ca^{2+}-activated K^+ channels. The first one to be discovered has been called charybdotoxin (Miller et al., 1985; Gimenez-Gallego, 1988). It blocks large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channels (Smith et al., 1986) and also several classes of voltage-sensitive K^+ channels (MacKinnon et al., 1988; Lewis et al., 1988; Schweitz et al., 1989; Stühmer et al., 1989). The second one has been initially called leiurotoxin I (Abia et al., 1986; Chicchi et al., 1988; Moczydlowski et al., 1988; Castle et al., 1989); it blocks a different class of small-conductance Ca^{2+}-activated K^+ channels which has also been shown to be the target of the bee venom toxin apamin (Hugues et al., 1982a,b,d; Blatz & Magleby, 1986; Castle & Strong, 1986; Bernardi et al., 1989; Auguste et al., 1990). Leiurotoxin I does not block charybdotoxin-sensitive K_{Ca} channels and vice versa. Because the name leiurotoxin I induces a possible confusion with classically called *Leiurus* toxins which block the voltage-sensitive Na^+ channel and also to mark clearly that charybdotoxin and leiurotoxin I block two different classes of K_{Ca} channels, we have previously proposed to rename leiurotoxin I scyllatoxin (ScyTx) (Charybdis and Scylla) (Auguste et al., 1990).

ScyTx is 31 amino acids long and has three disulfide bridges (Chicchi et al., 1988; Auguste et al., 1990). The main elements

† This study was supported by the Centre National de la Recherche Scientifique and the Ministère de la Défense Nationale (Grant DRET 90/192).
* To whom correspondence should be addressed.
‡ Institut de Pharmacologie Molêculaire et Cellulaire.
§ Institut Pasteur de Lille.

0006-2960/92/0431-648$03.00/0

of its spatial structure have been recently established by NMR (Martins et al., 1990). Like apamin, a toxin of 18 amino acids with two disulfide bridges (Shipolini, 1967), ScyTx blocks the postsynaptic hyperpolarization due to Ca^{2+}-activated K^+ channels in skeletal muscle cells in culture (Auguste et al., 1990) and contracts guinea pig taenia coli previously relaxed by epinephrine (Chicchi et al., 1988; Auguste et al., 1990). This is due to K_{Ca} channel blockade.

ScyTx inhibits in a competitive way the binding of ^{125}I-apamin to its receptor (Chicchi et al., 1988; Auguste et al., 1990). A synthetic analogue of ScyTx with a Tyr residue in position 2 (instead of Phe) has been prepared ([Tyr^2]ScyTx) and has been radioiodinated. ^{125}I-[Tyr^2]ScyTx identified a single class of sites on brain membranes with a K_d value of 80 pM. Apamin competitively inhibited ^{125}I-[Tyr^2]ScyTx binding (Auguste et al., 1990). These and other results strongly suggested that in spite of completely different sequences, apamin and ScyTx bind to the same receptor site on K_{Ca} channels.

The purpose of this work is 2-fold. It is first to establish the structure–function relationships of ScyTx and to identify amino acids in the sequence which are important for binding and activity. The second purpose of this work is to analyze whether all binding sites for apamin in different brain regions have the same or different affinities for ScyTx. If affinities are the same everywhere, this would suggest that there is only one type of apamin/ScyTx receptor associated with the sensitive K_{Ca} channel. If affinities are different, it would then be the indication of the existence of subtypes of apamin/scyllatoxin-sensitive K_{Ca} channels.

Experimental Procedures

Materials. 1,2-Cyclohexanedione, *O*-methylisourea, glycine ethyl ester, and water-soluble carbodiimide were purchased from Sigma; iodogene was from Pierce; and acetonitrile and trifluoroacetic acid (TFA) were from Merck.

Apamin Binding to Rat Brain Membranes. Mono-^{125}I-labeled apamin was purchased from NEN and was used in binding assays as described by Hugues et al. (1982a).

[Tyr^2]ScyTx Binding to Rat Brain Membranes. ^{125}I-[Tyr^2]ScyTx was obtained as previously described (Auguste et al., 1990). Competition experiments between ^{125}I-[Tyr^2]-ScyTx and modified toxins were performed as described by Auguste et al. (1990).

Biological Activity of Modified ScyTx. Effect on Guinea Pig Taenia Coli Contraction. The biological assay was carried out as described by Auguste et al. (1990).

Preparation of ScyTx Derivatives. Modification of arginine residues: Guanidine side chains of Arg_6 and Arg_{13} residues were modified by 1,2-cyclohexanedione according to Toi et al. (1967). A total of 100 nmol of synthetic ScyTx was incubated in a dark room with 0.2 N NaOH and 1 mg of 1,2-cyclohexanedione in a final volume of 1 mL. The reaction was stopped after 2 h with 10% TFA to obtain a final pH value of 2.5.

Guanidination: ϵ-Amino groups of lysines in position 20, 25, and 30 were transformed into homoarginine residues by reacting synthetic ScyTx with *O*-methylisourea hydrogen sulfate as described by Chauvet and Ascher (1967). A total of 100 nmol of synthetic ScyTx was incubated in 0.5 M methylisourea adjusted to pH 11 with 1 N NaOH. The reaction was stopped by lowering the pH to 2.5 with TFA after seven days of incubation at 4 °C.

Modification of carboxylate side chains: Carboxylate side chains of Asp_{24} and Glu_{27} were modified with glycine ethyl ester after activation of the carboxyl group by a water-soluble carbodiimide (Hoare & Koshland, 1967). A total of 100 nmol of synthetic ScyTx was incubated in a final volume of 200 μL with 1 M glycine ethyl ester and 100 mM water-soluble carbodiimide. The pH value was adjusted to 4 with 2 N HCl. After 30 min and after 90 min of incubation, 100 μmol of carbodiimide was added and the pH value was reequilibrated to 4. After a 24-h incubation, the pH was lowered to 2.5 with TFA.

Iodination: His_{31} was modified by iodination. A total of 25 nmol of synthetic ScyTx was incubated with 10 nmol of NaI and 15 nmol of iodogene in 50 nM Tris-HCl, pH 7.5, final volume 100 μL. After 15 min, the mixture was loaded onto a TSK-SP5PW column (75 × 0.75 cm) equilibrated with 50 mM MES, pH 6. Iodoscyllatoxin was eluted with a linear gradient of NaCl from 0 to 900 mM in 60 min. To better characterize monoiodoscyllatoxin, a radiolabeled ScyTx derivative was obtained by the same procedure using Na^{125}I.

Controls: In each particular case ScyTx used for controls was exposed to the same conditions (pH, temperature, etc.) as ScyTx undergoing chemical modifications without, of course, the key chemical reagents.

Purification of ScyTx Derivatives. After each type of chemical modification the reaction mixture was filtered on a cellulose acetate filter (0.2 μm, Corning) and loaded onto an HPLC column (Altex RPSC 4.6 × 75 mm) equilibrated with 0.1% TFA. Derivatives of ScyTx were eluted using a linear gradient of acetonitrile in 0.1% TFA from 0 to 40%, with a 0.75%/min slope.

Control of the Chemical Modifications. Modifications of Arg and carboxylate residues were monitored by Edman peptide sequencing on a gas-phase sequencer (Applied Biosystems 477A) equipped with an on-line phenylthiohydantoin (PTH) analyzer (Applied Biosystems 120A). Modifications of Lys residues were controlled using an amino acid analyzer.

Autoradiographic Procedures. Rats (Wistar, 150–200 g) were sacrificed, and their brains were removed, frozen in isopentane at −40 °C, and sectioned with a cryostat microtome. In equilibrium studies, the sections, 15 μm thick, were incubated for 30 min at 4 °C with 25 pM ^{125}I-apamin dissolved in 100 mM Tris-HCl buffer at pH 7.4 containing 0.1% bovine serum albumin. The nonspecific binding component was measured by adding a large excess of unlabeled apamin (1 μM) 15 min before adding labeled apamin. Competition binding experiments were performed by adding increasing concentrations (10 pM to 10 μM) of synthetic ScyTx in the presence of 25 pM ^{125}I-apamin. At the end of the incubation, sections were washed three times for 20 s in the buffer and once for 20 s in distilled water.

A part of the labeled sections was removed and counted in a γ counter. The other slides were dried and exposed to tritium-sensitive film (Hyperfilm ^{3}H, Amersham). Using an image-analysis computer (Samba, Alcatel-TITN), the autoradiographic investigations were carried out as previously described (Mourre et al., 1986). Specific binding values were determined as differences between total and nonspecific components for a given brain structure. Nomenclature used in this work followed that of Paxinos and Watson's atlas (1986).

Results

The structure of ScyTx is presented in Figure 1.

Purification of ScyTx Derivatives. Unmodified ScyTx eluted on a C_3 reverse-phase column with a retention time of 29.5 min (Figure 2). It could be easily separated from all ScyTx derivatives since they were more hydrophobic and eluted with longer retention times (33, 34.5, and 47 min for the Glu-modified, Arg-modified, and Lys-modified ScyTx deriv-

Ala - Phe - Cys - Asn - Leu - Arg - Met - Cys -
Gln - Leu - Ser - Cys - Arg - Ser - Leu - Gly -
Leu - Leu - Gly - Lys - Cys - Ile - Gly - Asp -
Lys - Cys - Glu - Cys - Val - Lys - His - NH_2

FIGURE 1: Amino acid sequence of C-terminal-amidated scyllatoxin.

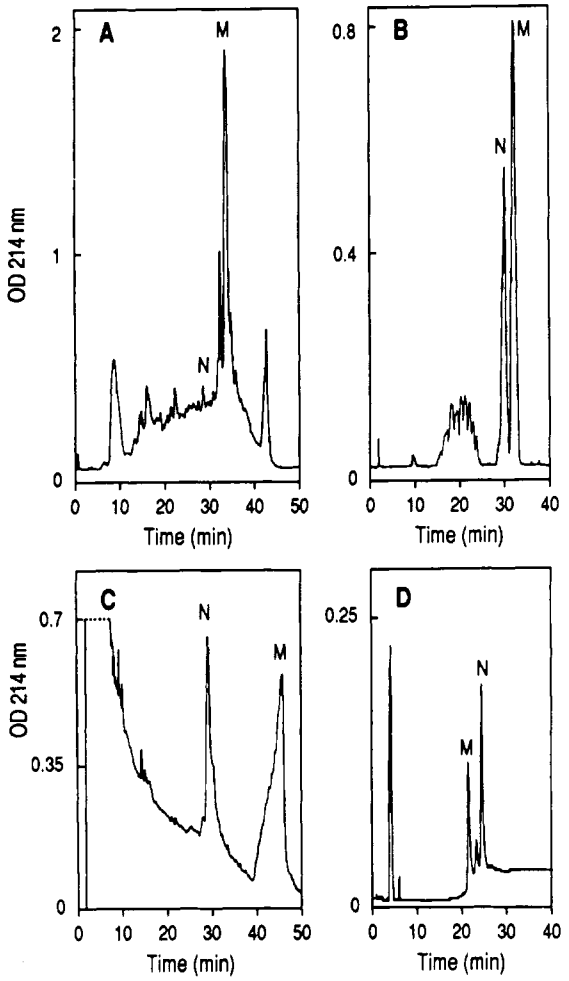

FIGURE 2: Purification of chemically modified derivatives of ScyTx on an Altex RPSC C_3 reverse-phase column (panel A, Arg-modified ScyTx; panel B, Glu-modified ScyTx; panel C, Lys-modified ScyTx) and on an ion-exchange (TSK-SP5PW) column (panel D, His-modified ScyTx). N, synthetic ScyTx; M, modified ScyTx.

atives, respectively; Figure 2).

Figure 2D shows a typical chromatogram describing the purification of the His-iodinated ScyTx. Iodinated ScyTx eluted at 320 mM NaCl, and synthetic ScyTx eluted at 390 mM NaCl.

Control of the Chemical Modifications. Chemical modification of Arg residues was checked by direct peptide sequencing. No PTH-Arg could be detected at cycle 6 of the peptide degradation under conditions in which the amount of PTH-Asn_5 was 342 pmol. Similarly, no PTH-Arg_{13} could be detected under conditions in which the amount of PTH-Ser_{14} was 372 pmol. These results indicate that >95% of the Arg_6 and Arg_{13} residues have been modified.

The same technique was used to assess the modification of Asp and Glu residues. No PTH-Glu_{27} could be detected under conditions in which 283 pmol of PTH-Val_{29} was detected. Recovery of PTH-Asp_{24} was 509 pmol. These results indicate that >95% of Glu_{27} residue has been modified under conditions which leave Asp_{27} residue unmodified.

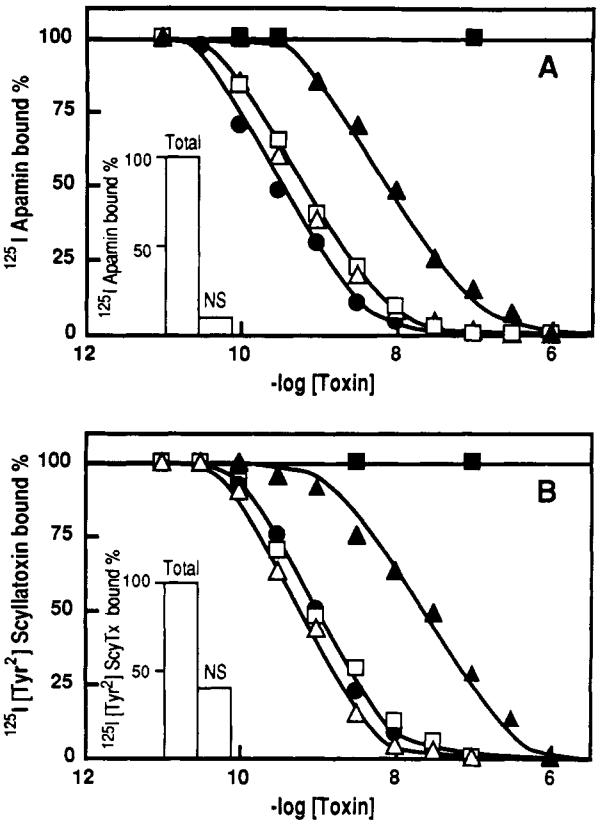

FIGURE 3: Inhibition of ^{125}I-apamin (20 pM) binding (A) and ^{125}I-ScyTx (150 pM) binding (B) to rat brain membranes (0.5 ng/mL) by increasing concentrations of synthetic ScyTx (●) and modified ScyTx: (△) Glu-modified ScyTx, (□) Lys-modified ScyTx, (▲) His-modified ScyTx, and (■) Arg-modified ScyTx.

Quantitative determinations of PTH-Lys are difficult. Therefore, modification of Lys residues was checked by amino acid analyses after acid hydrolysis. Under conditions in which 3.17 mol of Lys residue could be detected for the synthetic ScyTx, an identical concentration of Lys-modified ScyTx contained 0.07 mol of Lys residue/mol of toxin. These results indicate that 98% of the Lys residues have been modified.

His-modified ScyTx was detected on the chromatogram by comparison with ^{125}I-His-modified ScyTx (Figure 2). It could be prepared at a purity of >95%.

Effects on Binding Activity. Unlabeled synthetic scyllatoxin is known to inhibit ^{125}I-apamin and ^{125}I-[Tyr^2]ScyTx binding to brain membranes with affinity constants of 100 pM and 200 pM, respectively (Auguste et al., 1990). Scyllatoxin modified on its two Arg residues was completely unable to inhibit the binding of both ^{125}I-apamin and ^{125}I-[Tyr^2]ScyTx to their receptors (Figure 3). His_{31}-modified ScyTx was 40-fold less potent than ScyTx in inhibiting the binding of the two ^{125}I-labeled toxins to their receptors (Figure 3). However, modification of the Lys residues or of Glu_{27} did not alter ScyTx properties (Figure 3).

Effect on Taenia Coli. Scyllatoxin contracts taenia coli with an ED_{50} value of 6 nM (Chicchi et al., 1988; Auguste et al., 1990). The Arg_6-,Arg_{13}-modified ScyTx was totally inactive on taenia coli contraction even at concentrations as high as 1 μM (Figure 4). The ED_{50} value was 400 nM for a ScyTx with all of its Lys modified (Figure 4) instead of 6 nM for ScyTx. Glu_{27} modification changed the ED_{50} value from 6 to 200 nM. His_{31} modification induced a drastic loss of contractile potency with a ED_{50} value of 800 nM (Figure 4).

All of the data concerning binding and physiological studies of the different ScyTx derivatives are summarized in Table I.

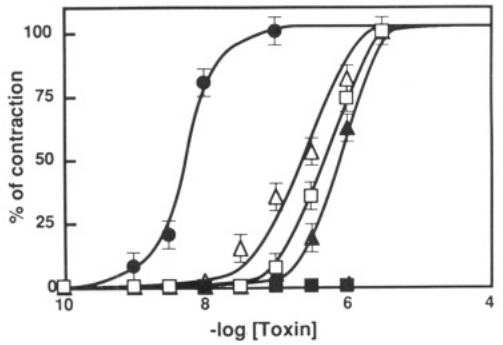

FIGURE 4: Effect of modified ScyTx on the contraction of guinea pig taenia coli previously relaxed by 1 μM epinephrine: (●) synthetic ScyTx, (△) Glu-modified ScyTx, (□) Lys-modified ScyTx, (▲) His-modified ScyTx, and (■) Arg-modified ScyTx. Experiments were performed in tyrode buffer.

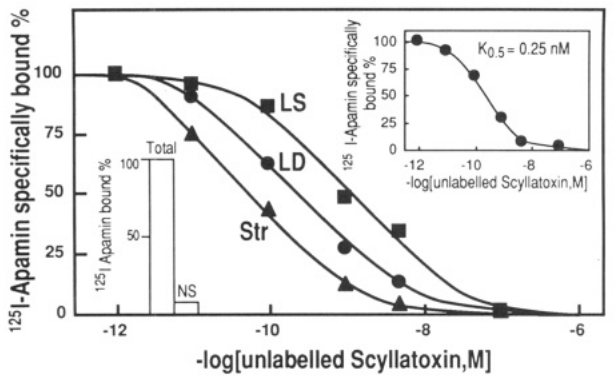

FIGURE 5: Quantitative autoradiographic determination of ^{125}I-apamin binding inhibition to rat brain sections by synthetic ScyTx: (■) LS, lateral septum ($K_{0.5}$ = 1100 pM); (●) LD, laterodorsal thalamic nucleus ($K_{0.5}$ = 220 pM); and (▲) Str, striate cortex ($K_{0.5}$ = 47 pM). Inset: γ counter determination of specific binding to a total brain section.

Table I: Effect of ScyTx and Modified ScyTx on ^{125}I-Apamin and ^{125}I-[Tyr^2]ScyTx Binding to Rat Brain Membranes and on Taenia Coli Contractions

amino acid modified	method	taenia coli contraction ED_{50} (nM)	inhibition of ^{125}I-apamin binding (K_d) (pM)	inhibition of ^{125}I-[Tyr^2]-ScyTx binding (K_d) (pM)
none		6 ± 1	100 ± 50	200 ± 50
$Arg_{6,13}$	cyclohexanedione	$>10^3$	$>10^5$	$>10^5$
$Lys_{20,25,30}$	*O*-methylisourea	400 ± 20	200 ± 50	200 ± 50
Glu_{27}	glycine ethyl ester	200 ± 10	160 ± 40	160 ± 40
His_{31}	iodination	800 ± 40	4000 ± 1500	8000 ± 2000

Effect of ScyTx on ^{125}I-Apamin Binding Sites in Brain Sections. ScyTx inhibited ^{125}I-apamin binding to brain sections (Figure 5, inset). The $K_{0.5}$ value (250 pM) was in good agreement with the value obtained with rat brain membranes (Auguste et al., 1990).

The heterogeneous distribution of ^{125}I-apamin receptors throughout rat brain has been previously described (Mourre et al., 1984, 1986). Very high densities of receptors were localized in habenula, lateral septum, neocortex, stratum oriens of hippocampus, molecular layer of dentate gyrus, and granular layer of cerebellar cortex. The basal ganglia and raphe presented low binding levels. Intermediate levels were found in the other brain areas (Figure 6) such as thalamus. The affinity of ^{125}I-apamin for its receptor in all parts of the brain corresponded to a K_d value of 25 pM.

Properties of inhibition of ^{125}I-apamin binding by various concentrations of ScyTx were found to be different brain

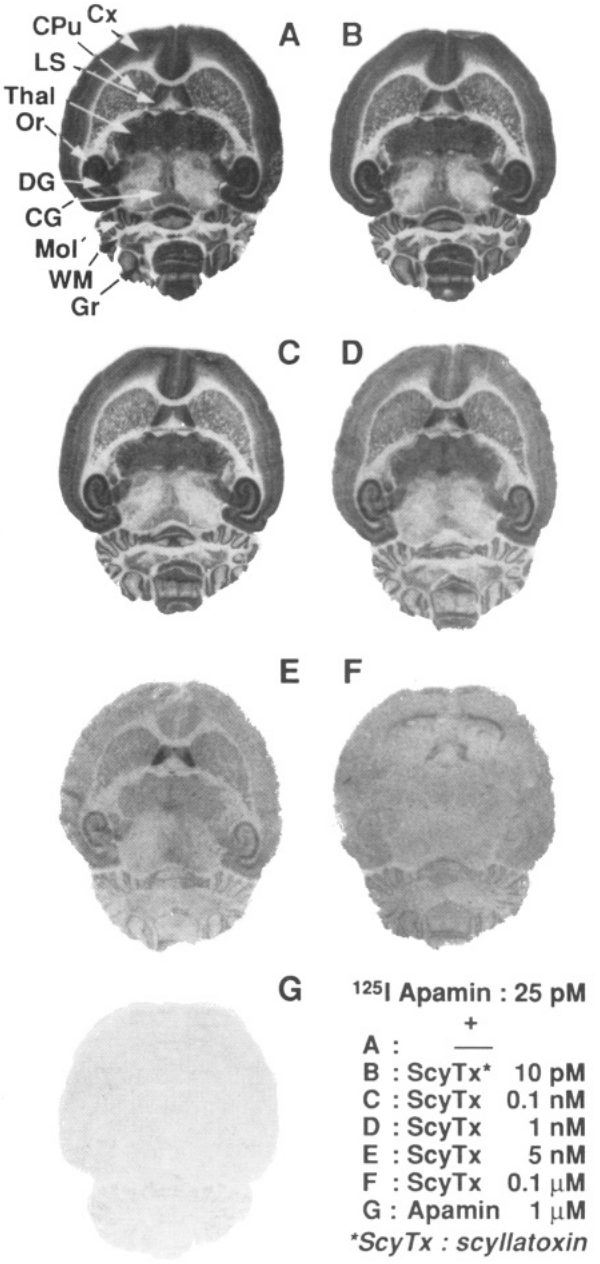

FIGURE 6: Localization of ScyTx binding sites in rat brain by autoradiographic analysis. Brain sections were incubated with 25 pM ^{125}I-apamin in the absence (A) or the presence of various concentrations of synthetic ScyTx (B–F) or unlabeled apamin (G). Abbreviations: CG, central grey; CPu, caudate putamen; Cx, neocortex; DG, dentate gyrus; Gr, granular layer of cerebellum; LS, lateral septum; Mol, molecular layer of cerebellum; Or, stratum oriens of Ammon's horn; Thal, thalamus; WM, white matter.

regions (Figure 6 and Table II). Three main groups of brain structures have been identified on the basis of their affinity for ScyTx. Group I has $K_{0.5}$ values lower than about 70 pM, group II corresponds to $K_{0.5}$ values between about 100 pM and 500 pM, and group III includes a $K_{0.5}$ value higher than 800 pM.

Figure 5 shows inhibition of ^{125}I-apamin binding by ScyTx for representative structures in each group. The $K_{0.5}$ value for the inhibition of ^{125}I-apamin binding by ScyTx was 47 pM for striate cortex, 220 pM for the laterodorsal thalamic nucleus, and 1100 pM for the lateral septum. These brain areas represent groups I, II, and III, respectively. Group II includes the largest number of brain regions (Table II). A relatively small number of regions, for the major part in neocortex, belong to group I. Only three structures, such as lateral

Table II: Autoradiographic Determination of the Inhibition of ^{125}I-Apamin Binding to Rat Brain Sections by ScyTx

rat brain section	$K_{0.5}$ (pM or nM)
Group I: 30 pM < $K_{0.5}$ < 70 pM	
basolateral amygdaloid nucleus	35 ± 21
nucleus of the vertical limb of the diagonal band	40 ± 10
striate cortex	47 ± 10
temporal cortex, auditory area	54 ± 15
ventral tegmental area	64 ± 14
central medial thalamic nucleus	65 ± 13
parietal cortex	69 ± 16
medial septal nucleus	72 ± 16
Group II: 100 pM < $K_{0.5}$ < 500 pM	
superficial gray layer of the superior colliculus	102 ± 10
entorhinal cortex	103 ± 12
granular layer of the cerebellar cortex	105 ± 12
occipital cortex	110 ± 13
accumbens nucleus	130 ± 70
ventromedial thalamic nucleus	130 ± 46
ventral posterolateral thalamic nucleus	140 ± 150
molecular layer of the cerebellar cortex	140 ± 60
hilus	140 ± 20
molecular layer of the dentate gyrus	150 ± 14
inferior colliculus	170 ± 95
stratum lacunosum moleculare of the Ammons's horn	170 ± 25
caudate putamen	180 ± 11
primary olfactory cortex	180 ± 79
stratum radiatum of the Ammons's horn	190 ± 13
substantia nigra	210 ± 35
ventral cochlear nucleus	210 ± 77
laterodorsal thalamic nucleus	220 ± 80
central gray	220 ± 134
stratum pyramidal of the Ammons's	220 ± 27
stratum oriens of the Ammons's horn	240 ± 109
granular layer of the dentate gyrus	240 ± 100
medial geniculate nucleus	250 ± 180
deep mesencephalic nucleus	250 ± 112
dorsal lateral geniculate nucleus	330 ± 64
interpositus cerebellar nucleus	330 ± 90
vestibular nuclei	380 ± 101
corpus callosum	430 ± 109
lateral hypothalamic area	480 ± 103
pontine reticular nuclei	520 ± 127
Group III: 0.8 nM < $K_{0.5}$ < 1.2 nM	
spinal tract of the trigeminal nerve	0.81 ± 0.17
lateral septum	1.1 ± 0.12
lateral preoptic area	1.2 ± 0.1

septum, composed group III (Table II).

Discussion

2D NMR studies have indicated that ScyTx comprises an α-helical region near the N-terminal part (Arg_6–Arg_{16}) and an antiparallel β-sheet near the C-terminal end of the molecule (Leu_{18}–Val_{29}) (Martins et al., 1990). In addition, the structure is of course maintained by the three disulfide bridges, the integrity of which is essential for the toxin activity (Auguste et al., 1990).

It has been shown in this work that the two arginines in the ScyTx structure (Arg_6 and Arg_{13}) are essential both for the binding activity to the apamin/ScyTx receptor and for the contractile action on taenia coli taken as an index of the activity on K_{Ca} channels. This observation is interesting and not really totally unexpected since the most important amino acids for apamin function are also two arginines (Cosand & Merrifield, 1977; Granier et al., 1978; Hugues et al., 1982a; Vincent et al., 1975; Labbé-Jullié et al., 1991), both situated in an α-helical structure (Pease & Wemmer, 1988). However, in apamin, these arginines, Arg_{13} and Arg_{14}, are in contiguous positions. Arg_6 and Arg_{13} in ScyTx are not contiguous, but they are clearly close to each other in the spatial structure (Martins et al., 1990).

Another very important amino acid for ScyTx function is the C-terminal histidine residue in position 31. Iodination of this residue leads to a 40-fold decrease in the binding activity toward brain receptors and to a decrease of about 2 orders of magnitude in the ED_{50} for the contractile activity on taenia coli. The importance of this residue for the interaction of ScyTx with the K_{Ca} channel has practical consequences. It makes it impossible to directly ^{125}I iodinate the native toxin for identification of the receptor structure. It is why we previously had to incorporate a Tyr residue in the synthetic sequence (Auguste et al., 1990).

Of the three lysine residues, two of them, Lys_{20} and Lys_{25}, are situated within the sequence that form the β-sheet structure. The other one, Lys_{30}, is between the end of this β-sheet structure and His_{31} which, as we have just seen, is very important for the toxin activity. Our results clearly show that transformation of these lysine residues into homoarginines does not significantly change the interaction of the modified toxin with the brain apamin ScyTx receptor (Table I). However, this modification drastically alters (a factor of about 70) the contraction inducing properties of the toxin. This could be due to the fact that although Lys modification does not alter the interaction of ScyTx with its brain sites, it alters the toxin interaction with the taenia coli binding sites. The other and more probable interpretation is that the binding is not altered but that its functional expression is deteriorated. It is, of course, not known which of the Lys residues is or are most essential for the expression of the intestinal contractile activity. Such a question will only be answered by appropriate substitution of each Lys residue in synthetic analogues of the natural toxin.

Asp_{24} belongs to the β-turn structure. Its side chain could not be modified and is then probably not reactive and involved in ionic interaction with the Lys_{25} residue. Glu_{27} is also part of the antiparallel β-sheet structure. Its carboxylic side chain was rapidly modified without any significant effect on the binding activity to brain receptors (Table I) but with a significant decrease (a factor of about 30) of the toxin capacity to contract taenia coli. Once again, as for the lysine residues, Glu_{27} might be primarily involved in a functional step following toxin binding which would be essential for K_{Ca} channel blockade. All these results taken together would suggest that the α-helical structure with its two Arg as well as the C-terminal His is an essential element for binding to the K_{Ca} channel protein whereas the β-sheet structure could be more specifically involved in a step following binding and important for K_{Ca} channel blockade.

The three-dimensional structure of charybdotoxin was established by NMR (Bontems et al., 1991) shortly after the structure of ScyTx (Martins et al., 1990). Charybdotoxin (37 residues, three disulfide bridges) and ScyTx (31 residues, three disulfide bridges) have the same type of folding. Charybdotoxin, like ScyTx, presents a short helix near the N-terminal part, a C-terminal antiparallel β-sheet, and three disulfide bridges. It is then tempting to propose that the main elements of the active site of charybdotoxin are situated on its α-helical structure. Charybdotoxin α-helical structure contains two positively charged amino acids (Lys_{11} and Arg_{19}), which could be involved in charybdotoxin blockade of large-conductance K_{Ca} channels. The charybdotoxin β-sheet structure contains two lysines (Lys_{27} and Lys_{32}). One can find an analogous structure with ScyTx where Lys_{20} and Lys_{25} are located in the β-sheet part of the toxin. It is probably not a coincidence since other residues nearby are different (although there are two additional positive charges on Lys_{31} and Arg_{34}). It would not

be surprising again if these lysines were also contributing to the charybdotoxin activity.

Glu_{27} is replaced by an arginine in the charybdotoxin sequence. His_{31} has no equivalent in charybdotoxin, which has the C-terminal sequence is Cys-Tyr-Ser instead of Cys-Val-Lys-His. This residue is probably structurally immobilized and important for the spatial structure of the toxin since it cannot be removed from the non-amidated form of ScyTx (see Figure 1) by carboxypeptidase A (not shown).

Quantitative autoradiography is a particularly efficient technique for analyzing mutual interactions in different parts of the brain of different types of toxins associated with one given class of ionic channels. This was particularly evident for two polypeptide toxins specific for voltage-dependent K^+ channels, one from bee venom MCD peptide (MCD), and the other one from snake venom dendrotoxin I (DTX_I) (Bidard et al., 1989).

This work has shown that high-affinity apamin receptors (K_d = 25 pM) do not always have a high affinity for ScyTx (Table II). Some of these receptors have a very high apparent affinity ($K_{0.5}$ = 47 or 35 pM) for ScyTx, such as neocortex and amygdala, others have a modest apparent affinity ($K_{0.5}$ = 1.1 nM) as in lateral septum. Intermediate affinities ($K_{0.5}$ between 100 and 500 pM) could also be observed as in laterodorsal thalamic nucleus. However, the inhibition curves appear rather flat and could suggest the presence of multiple sites in the structure studied.

It is then clear that apamin-sensitive K_{Ca} channels in the brain constitute a family of channels with different affinities for ScyTx. The same situation has been previously observed for voltage-dependent K^+ channels. In that case, high-affinity DTX_I receptors have been found to display high, intermediate, or low affinity for MCD (Bidard et al., 1989). Proportions of high-, intermediate-, and low-affinity sites associated to high-affinity DTX_I binding site have been found to vary in different brain areas. Different subtypes of voltage-sensitive Na^+ channels have also been found to express different types of affinities (high, intermediate, or low) for tetrodotoxin (Mourre et al., 1987; Lombet et al., 1982) or for sea anemone toxins (Frelin et al., 1984).

Affinity labeling experiments have suggested that apamin-sensitive K_{Ca} channels are constituted by different types of subunits with molecular masses between 86 kDa and 23 kDa (Hugues et al., 1982c; Seagar et al., 1986; Auguste et al., 1989). However, the exact stoichiometry of these subunits is not known and none of them has yet been cloned. It may be that a diversity of subunit composition in different part of the brain will lead to a diversity of affinities for ScyTx as it is for voltage-sensitive K^+ channels and their interaction with DTX_I and MCD (Bidard et al., 1989; Stühmer et al., 1989).

ACKNOWLEDGMENTS

We thank C. Widmann, C. Roulinat, and F. Aguila for very skillful technical assistance.

Registry No. ScyTx, 116235-63-3; apamin, 24345-16-2.

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Solution Structure of the Lipophosphoglycan of *Leishmania donovani*†

S. W. Homans,*,‡ A. Mehlert,‡ and S. J. Turco§

Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K., and Department of Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky 40536

Received May 16, 1991; Revised Manuscript Received October 10, 1991

ABSTRACT: The three-dimensional solution structure of the repeating $-PO_4-6Gal\beta1-4Man\alpha1-$ disaccharide fragment of the lipophosphoglycan (LPG) derived from *Leishmania donovani* has been determined by use of a combination of homo- and heteronuclear NMR spin coupling constant measurements together with restrained molecular mechanical minimization and molecular dynamics simulations. The fragment exists with limited mobility in solution about the Galβ1-4Man linkages, whereas in contrast a variety of stable rotamers exist about the Manα1-PO_4-6Gal linkages. These rotamers define several major stable conformers in solution, which are discussed in terms of the proposed biological role of LPG.

The trypanosomatid *Leishmania donovani* is the etiologic agent of kala azar. It proliferates in hydrolytic environments throughout its digenetic life cycle: as an extracellular promastigote in the alimentary canal of its sandfly vector and as an intracellular amastigote within the phagolysosomes of mammalian macrophages. A major cell surface component of *L. donovani* is a unique glycoconjugate called lipophosphoglycan (LPG) (King et al., 1987). Structurally, the *L. donovani* LPG is a polymer of repeating phosphorylated disaccharide units (on average 16 repeats) of PO_4-6Galβ1-4Manα1 linked via a hexasaccharide carbohydrate core to a lyso-1-*O*-alkylphosphatidylinositol lipid anchor (Orlandi & Turco, 1987; Turco et al., 1987, 1989). A similar but immunologically distinct glycoconjugate has been reported in all species of *Leishmania* (Hernandez, 1983; Handman et al., 1984; Handman & Goding, 1984; McConville et al., 1987, 1990). LPG has been reported to bind Ca^{2+} (Eilam et al., 1985) and the biological significance of this is unknown.

The three-dimensional structures of the LPGs from *Leishmania* species are of potential importance since they may help to explain the multiple functions that have been proposed [reviewed by Turco (1990)]. LPG is believed to be required for attachment of promastigotes to the epithelial cells that line the midgut of the sandfly (Davies et al., 1990). Following inoculation of the parasites by the sandfly, LPG promotes complement activation and resistance to complement-mediated damage in the bloodstream of the host (Puentes et al., 1988). LPG also plays a key role in the receptor-mediated attachment and entry into mammalian macrophages (Handman & Goding, 1984; Da Silva et al., 1989; Talamas-Rohana et al., 1990). Upon entry within host macrophages, LPG has been shown to be required for intracellular survival (Handman et al., 1986; Elhay et al., 1990; McNeely & Turco, 1990). The precise intracellular role (or roles) of LPG within phagolysosomes is unclear. There is evidence that the glycoconjugate may interact with the host cell's protein kinase C (McNeely & Turco, 1987; McNeely et al., 1989), thereby preventing activation of the microbicidal oxidative burst. LPG also might act as a chelator of divalent cations (i.e., Ca^{2+} or Fe^{2+}) that are necessary to sustain the burst and/or behave as a scavenger of toxic oxygen metabolites (Chan et al., 1989; McNeely & Turco, 1990). Thus, the delineation of the three-dimensional structure of the glycan represents a major step in our understanding of the mechanism of LPG oligosaccharide–host molecule interactions. Furthermore, the demonstration that vaccination of mice with purified *Leishmania major* LPG induces resistance against cutaneous leishmaniasis (Handman & Mitchell, 1987; McConville et al., 1987) suggests that it might be possible to generate synthetic vaccines from structural homologues of LPG.

Here, by use of a combination of homo- and heteronuclear NMR spin coupling constant measurements together with restrained molecular mechanical minimization and molecular dynamics simulations, we have determined the structural and dynamic properties of the LPG from *L. donovani* and have investigated the effect of Ca^{2+} ions on the 3D structure. The significance of these data is discussed in terms of the proposed biological role of this glycoconjugate.

Experimental Procedures

Purification of LPG. LPG from *L. donovani* promastigotes was extracted and partially purified as described earlier

† Financial support was provided from The Wellcome Trust (S.W.H.), from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, and from NIH Grant AI20941 to S.J.T. S.J.T. is a Burroughs Wellcome Scholar in Molecular Parasitology.
‡ University of Dundee.
§ University of Kentucky Medical Center.

0006-2960/92/0431-654$03.00/0