Distinct primary structures of the major peptide toxins from the venom of the spider *Macrothele gigas* that bind to sites 3 and 4 in the sodium channel[1]

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Abstract **Six peptide toxins (Magi 1–6) were isolated from the Hexathelidae spider *Macrothele gigas*. The amino acid sequences of Magi 1, 2, 5 and 6 have low similarities to the amino acid sequences of known spider toxins. The primary structure of Magi 3 is similar to the structure of the palmitoylated peptide named PlTx-II from the North American spider *Plectreurys tristis* (Plectreuridae). Moreover, the amino acid sequence of Magi 4, which was revealed by cloning of its cDNA, displays similarities to the Na^+ channel modifier δ-atracotoxin from the Australian spider *Atrax robustus* (Hexathelidae). Competitive binding assays using several ^{125}I-labelled peptide toxins clearly demonstrated the specific binding affinity of Magi 1–5 to site 3 of the insect sodium channel and also that of Magi 5 to site 4 of the rat sodium channel. Only Magi 6 did not compete with the scorpion toxin LqhαIT in binding to site 3 despite high toxicity on lepidoptera larvae of 3.1 nmol/g. The K_is of other toxins were between 50 pM for Magi 4 and 1747 nM for Magi 1. In addition, only Magi 5 binds to both site 3 in insects (K_i = 267 nM) and site 4 in rat brain synaptosomes (K_i = 1.2 nM), whereas it showed no affinities for either mammal binding site 3 or insect binding site 4. Magi 5 is the first spider toxin with binding affinity to site 4 of a mammalian sodium channel.**

Key words: Spider; Peptide; Toxin; Insecticide; Structure; Pharmacology; Ion channel

1. Introduction

Natural drugs that modulate ion channels represent a key class of pharmaceutical agents across many therapeutic areas and there is considerable potential for development of drugs effective to ion channels [1]. To study the interaction of natural ligands with ion channels, high binding affinity ligands are the best chemical probes to enable biochemical assays when using limited amounts of ion channel preparations. Moreover, small molecular weight natural ligands are generally preferred for ease of chemical synthesis, which avoids natural extractions. Spider venoms contain strong peptide ligands with a variable range of molecular masses (3–12 kDa), including a large diversity of ion channel antagonists comparable to the venoms of other animals [2]. Therefore, spider ion channel toxins are good candidates for the study of ion channels. Several spider toxins targeting different types of mammalian calcium channels and subtypes of potassium ion channels have been identified: ω-AgaIVA (P-type), ω-grammotoxin (N-type), SNX-482 (R-type), hanatoxin (Kv2.1), heptarotoxins (Kv4.2) (for a recent review [3]). Spider peptide toxins modifying sodium channels have also been characterized. δ-Atracotoxins from the Australian spiders *Atrax robustus* [4] and *Hadronyche versuta* [5] bind to site 3 of insect and mammal sodium channels [6]. Moreover, palutoxins and ProTxs were isolated from the venom of the amaurobiid spider *Paracoelotes luctuosus* and of the theraphosid spider *Thrixopelma pruriens*, respectively [7,8]. Palutoxins block inactivation of insect sodium channels [7], and ProTxs are antagonists of rNav 1.8 sodium channels [8]. Most of the small molecular weight ion channel toxins from spider venoms have been successfully synthesized, and applied for pharmacological and structural studies of ligand–receptor interactions [8–10]. Moreover, some synthetic spider toxins have been used for studies of ligand–receptor recognition in calcium and potassium ion channels using ion channel mutants [9,11,12]. Other studies on the molecular interaction of ligand and receptor have employed synthetic mutants of ion channel ligands [13–15]. Therefore, small molecular weight high affinity ion channel ligands from spider venoms could be good candidates for this purpose.

Searching for small molecular weight peptide ligands affecting sodium ion channels, we report the isolation, chemical and biochemical characterization of five peptides that bind to site 3 and site 4 of the sodium channel, and one peptide with unknown mode of action from the venom of the hexathelid spider *Macrothele gigas*.

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[1] The protein sequences reported in this paper have been submitted to the Swiss Protein Database under SwissProt accession codes P83557 for Magi 1, P83558 for Magi 2, P83559 for Magi 3, P83560 for Magi 4, P83561 for Magi 5, and P83562 for Magi 6. The nucleotide sequence reported in this paper is available in DDBJ/EMBL/GenBank™ databases under accession number AB105149.

Abbreviations: TFA, trifluoroacetic acid; PC, protease convertase; ED_{50}, peptide dose that paralyzes 50% of the animals; LD_{50}, peptide dose that kills 50% of the animals

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2. Material and methods

2.1. Biological materials

M. gigas venom was obtained by electrical stimulation of field-collected spiders from the Sonai area in Iriomote, Japan. The crude venom was frozen and stored at −20°C until use. Endoproteinase Glu-C (EC 3.4.21.19) was from Sigma (USA).

2.2. Isolation, amino acid sequence and mass spectrometry analysis of M. gigas toxins

The crude venom (70 μl) was resuspended in 0.1% aqueous trifluoroacetic acid (TFA) containing 10% acetonitrile (CH_3CN), and the insoluble material was removed by centrifugation at 14 000 × *g* for 5 min. The supernatant was fractionated directly by established high performance liquid chromatography (HPLC) methods [16]. The amino acid sequences and the mass spectra of the six major peptide toxins from the whole venom were analyzed according to previously published procedures [7].

2.3. Biological activity

Biological activity in insects (*Spodoptera litura*, tobacco cutworm) and mice (C57/Bl6) was evaluated according to previously described assays [7]. Negative controls were done with saline solution, and positive controls were performed with a toxic scorpion peptide LqhIV isolated in our laboratory [17].

2.4. Neuronal membrane preparations

All buffers contained a cocktail of proteinase inhibitors composed of phenylmethylsulfonyl fluoride (50 μg/ml), pepstatin A (1 μM), iodoacetamide (1 mM) and 1,10-phenanthroline (1 mM). Insect and rat brain synaptosomes were prepared from whole heads of adult cockroaches of *Periplaneta americana* and albino Sprague–Dawley rats, respectively, according to a previously described method [18]. No loss of binding activity was observed after 6 months at −80°C. Membrane protein concentration was determined using a Bio-Rad protein assay, using bovine serum albumin (BSA) as standard.

2.5. Iodination of δ-TxVIA, LqhαIT, BjxtrIT, Lqh2 and CssIV

δ-TxVIA was iodinated as described [19]. The toxins were radioiodinated by 0.7 units of lactoperoxidase, 1 nmol of toxin and 0.5 mCi carrier-free $Na^{125}I$ (Amersham, UK). The mono-iodotoxins were purified using an analytical Vydac reverse phase (RP) HPLC C18 column with a gradient of buffers A (H_2O, 0.01% TFA) and B (acetonitrile, 0.01% TFA) and eluted just after the non-modified toxin. The gradient was from 0 to 15% of buffer B in 5 min, then from 15% to 40% in 120 min. The concentration of the radiolabeled toxin was determined according to the specific activity of the ^{125}I corresponding to 2500–3000 dpm/fmol of mono-iodotoxin, depending on the age of the radiotoxin and by estimation of its biological activity.

2.6. Binding assays

Standard binding medium composition was: choline-Cl, 130 mM; $CaCl_2$, 1.8 mM; KCl, 5 mM; $MgSO_4$, 0.8 mM; HEPES, 50 mM; glucose 10 mM; and 2 mg/ml BSA. Wash buffer composition was: choline-Cl, 140 mM; $CaCl_2$, 1.8 mM; KCl, 5.4 mM; $MgSO_4$. 0.8 mM; HEPES, 50 mM; 5 mg/ml BSA, pH 7.5. Insect (3–8 μg of protein/ml) and rat (0.4–1.0 mg/ml) membrane preparations were suspended in 0.2 ml binding buffer, containing iodinated toxins. After incubation for 60 min, the reactions were terminated by dilution with 2 ml ice-cold wash buffer. Separation of the free from bound toxin was achieved under vacuum by rapid filtration through GF/C filters (Whatman, Maidstone, UK) preincubated in 0.3% polyethyleneimine (Sigma, USA). The filters were then rapidly washed twice with 2 ml of buffer. Non-specific toxin binding was determined in the presence of a high concentration of the unlabeled toxin and consisted typically of 5–35% of total binding. Competition binding experiments were analyzed using the computer program KaleidaGraph (Synergy Software, Reading, MA, USA) using a non-linear Hill equation (for IC_{50} determination). The K_is were calculated by the equation $K_i = IC_{50}/(1+(L^*/K_d))$, where L^* is the concentration of hot toxin and K_d is its dissociation constant [20].

2.7. Amplification of total cDNA and characterization of the partial Magi 4 cDNA ends

The total RNA was prepared (from two venom glands of a female spider) using TRIzol reagent according to the manufacturer's protocol (Invitrogen, San Diego, CA, USA). The first strand cDNA was synthesized using Powerscript Reverse Transcriptase, 3′SMART CDS primer II, and SMART II A oligonucleotides according to the manufacturer's protocol (BD Bioscience, Palo Alto, CA, USA), and was amplified by polymerase chain reaction (PCR) with 5′PCR primer II A and Advantage 2 polymerase (BD Bioscience), consisting of 95°C for 1 min and 24 cycles of 95°C for 5 s, 65°C for 5 s, and 68°C for 6 min on a thermal cycler (Model GeneAmp PCR system 9600; PE Biosystems, Foster City, CA, USA). All PCR amplifications described below were performed using EX-Taq polymerase (Takara Shuzo, Kyoto, Japan) or rTaq DNA polymerase (Toyobo, Osaka, Japan). The first-round PCR was performed with the amplified total cDNA as templates, the oligo-dT anchor primer (Roche Diagnostics, Basel, Switzerland) and the degenerate primers 5′-TG(T/C)GGIIIIAA(A/G)(A/C)GIGCITGGTG(T/C)AA-3′ (I represents inosine) corresponding to the sequence Cys^1–Lys^9. The first PCR products reamplified with the anchor primer (Roche Diagnostics) and the degenerate primers, 5′-GA(A/G)AA(A/G)AA(A/G)GA(T/C)TG(T/C)TG(T/C)TG(T/C)GG, corresponding to Glu^{10}–Gly^{17}. Both first- and second-round PCR consisted of 95°C for 30 s and five cycles of 95°C for 15 s, 45°C for 30 s, and 72°C for 1 min followed by 30 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 1 min (10 min in the last cycle). The second-round PCR products were subcloned into TOPO TA-cloning vector (Invitrogen, San Diego, CA, USA), and the inserts were amplified by PCR with M13 universal primers.

2.8. Determination of the 5′-end sequence of Magi 4 cDNA and DNA sequencing

The amplified cDNA was dA-tailed with dATP and terminal transferase (Roche Diagnostics), and was subjected to PCR with the oligo-dT anchor primer and a gene-specific primer (GTTACGTATTTTTAATACA, complementary to nucleotides 457–476), consisting of 95°C for 30 s and 35 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min. The PCR products were subcloned and the inserts were amplified as described above. All nucleotide sequences were determined with Big-Dye sequencing kit ver.1.1 and a model 373 DNA sequencer (PE Biosystems), and then analyzed with GENETYX-MAC software (Software Development, Tokyo, Japan).

3. Results

3.1. Purification and chemical analysis of M. gigas major peptides

The crude venom of both female and male species of *M. gigas* contains similar peptide composition. However, two extra peptides were found in the crude venom of the male spiders at *m/z* of 3177.9 and 3270.1 indicating some minor differences between male and female spiders (Fig. 1A). Since the quantity of venom extracted was higher for the female spiders than for the male spiders, only female spider venom was pooled and fractionated using RP-HPLC (Fig. 1B). Eighty fractions were manually collected and evaporated under vacuum conditions. Because of the small supply of venom, only the major peak fractions of the spider venom were selected for chemical and biological analysis. The six larger peptide fractions (49, 51, 54, 55, 63 and 69) were further fractionated by cation exchange chromatography. All six fractions yielded a major peak containing a single molecule as observed by mass spectrometric analysis. To ensure purity, the six peptides were finally purified to homogeneity by RP-HPLC using a C4 column. The molecular masses of the major single molecules were 4563.0, 4940.3, 5222.8, 5150.0, 3286.8 and 4036.4 Da, respectively. The purity of all six peptides was further confirmed by capillary zone electrophoresis. The molecules were named Magi 1–6 corresponding to the fractions 49, 51, 54, 55, 63 and 69, respectively according to their elution times under RP-HPLC conditions. Magi 1 was the most hydrophilic molecule of all six since it had the earliest elution time under

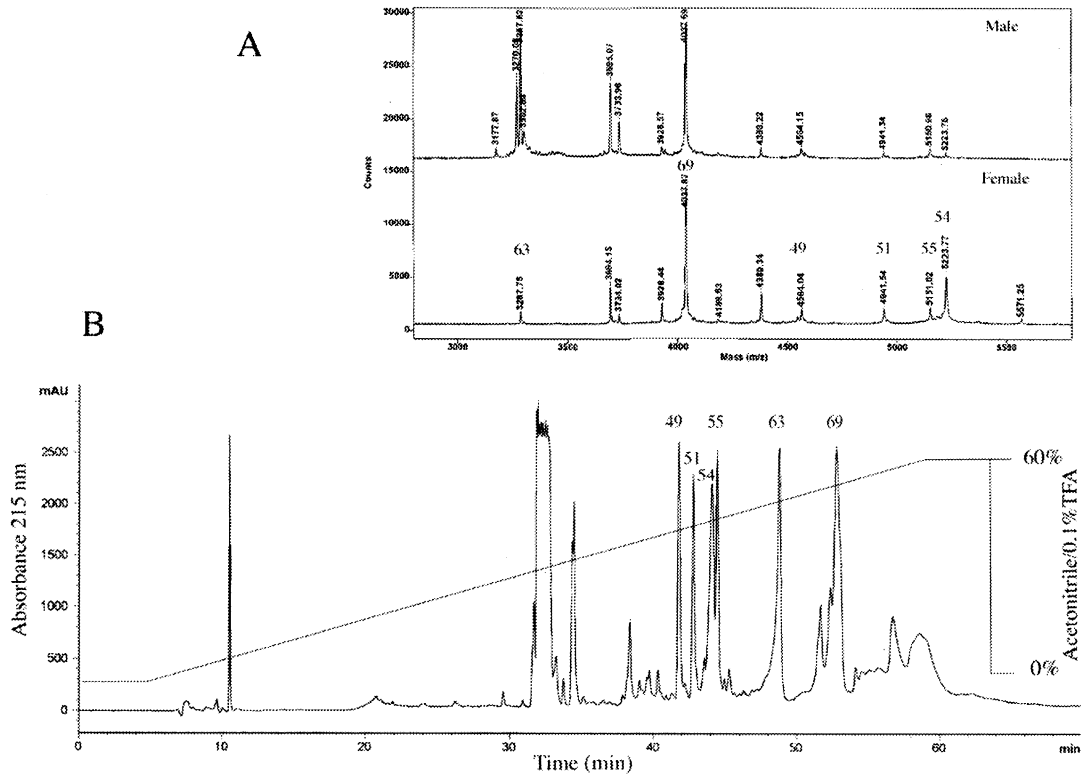

Fig. 1. A: Mass spectrum of the crude venom of male and female *M. gigas*. B: RP-HPLC chromatogram of the crude female venom of *M. gigas*. The numbers 49, 51, 54, 55, 63 and 69 represent the HPLC fractions containing the six major peptide toxins named Magi 1, 2, 3, 4, 5 and 6, respectively in A (mass/charge ratio) and B (retention times under RP-HPLC conditions).

RP-HPLC. A mass spectrometric analysis of the six peptides before and after alkylation indicated that Magi 1, 2 and 5 contained three disulfide bridges, Magi 4 and 6 contained four disulfide bridges, and Magi 3 contained five disulfide bridges. Direct Edman degradation of five out of six alkylated peptides allowed unequivocal identification of their first 29–35 amino acid residues. The complete amino acid sequences of Magi 1, 2, 3, 5 and 6 were obtained after their enzymatic digestion with chymotrypsin and subsequent Edman degradation of their individual enzymatically digested fragments. Magi 4, the peptide with the lowest concentration of the six in the spider venom, yielded only 23 residues and its enzymatic cleavage with lysine-C produced an internal fragment of seven additional amino acid residues. Magi 4 was further characterized by isolation of its mRNA from dissected venom glands.

3.2. *cDNA characterization and complete amino acid sequence of Magi 4*

To verify the full-length sequence of Magi 4, we initially tried to clone the Magi 4 cDNA by rapid amplification of cDNA ends (RACE) experiments. However, this attempt did not succeed, most likely because of too small amount of total RNA from a limited number of samples. Thus, we amplified whole cDNA of the spider venom employing the SMART PCR cDNA system (see Section 2), and then applied the resultant cDNA to the first-round PCR using degenerate primers corresponding to Cys^{1}–Lys^{9} in the Magi 4 fragment followed by the nested PCR with the degenerate primers encoding the sequence Glu^{10}–Gly^{17}. Electrophoresis of the nested-round PCR mixture revealed the amplification of a major PCR product of ~0.25 kb (data not shown). Sequencing of the subcloned inserts demonstrated that all clones comprised essentially identical nucleotide sequences apart from minor differences in the 3′-terminal sequence, due to various lengths of the poly(A) tracts. The predicted amino acid sequence showed that the sequence YNCVYA, which accorded with the internal peptide fragment Tyr^{18}–Ala^{23} in the partial amino acid sequence of Magi 4 obtained by Edman degradation, was located immediately after the sequence derived from

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                                                GAAGCAGTGGTATCAACGCAGAGTACGC   28
GGGGAGAGCAAGTTCAGAAACAGATCCTTGCACAGGAAGTCTCTGCTTACGTCTCCGAAT   88
ATGAAGACCTTAGTGATTGCTTGCGTTGCTTTGGTGCTGGTGGTTGTACATGGCGAGGTG  148
 M  K  T  L  V  I  A  C  V  A  L  V  L  V  V  V  H  G  E  V   20
ATTGAAGAAGTGAATGAAAAACAACTTCAAGAAAGTGTGGAAGAGAAGTACTCACTTTTG  208
 I  E  E  V  N  E  K  Q  L  Q  E  S  V  E  E  K  Y  S  L  L   40
CAGAGACTGGAAAAGCTTGACGAAGCTATCACGGCAGAAGAAAACAGAAACTCTCGTGTA  268
 Q  R  L  E  K  L  D  E  A  I  T  A  E  E  N  R  N  S  R  V   60
AGGCGTTGCGGCAGCAAGAGAGCTTGGTGTAAGGAAAAGAAAGACTGCTGCTGCGGATAT  328
 R  R  C  G  S  K  R  A  W  C  K  E  K  K  D  C  C  C  G  Y   80
AACTGCGTCTATGCCTGGTACAATCAACAGTCTTCTTGCGAAAGGAAATGGAAATATCTT  388
 N  C  V  Y  A  W  Y  N  Q  Q  S  S  C  E  R  K  W  K  Y  L  100
TTCACAGGTGAGTGCTAAAATACCTGAAAACGGCGAACGACATTCTTTAAAATCTTAAAA  448
 F  T  G  E  C  *                                             105
AAGAAAATTGTATTAAAAATTACGTAACTTTGTCAAGCAGTATTCTGCTAAAGAAGCTTC  508
ATTAAAATTTTATGAAGACCTAAAAAAAAAAAAAAAA                         538
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Fig. 2. A cDNA and deduced amino acid sequence of Magi 4 peptide toxin. The complete amino acid sequence of Magi 4 is underlined.

degenerate primers. This result suggested that the cDNA clone encoded the precursor of Magi 4. In an attempt to determine the complete cDNA sequence, we performed 5′-RACE with the primers specific for the clone. A 0.4 kb single clone was obtained and sequenced after subcloning, revealing that the PCR product encoded an amino acid sequence that completely agrees with the sequence of the Magi 4 fragments obtained by Edman degradation. In addition, PCR products amplified using different polymerases contained the identical nucleotide sequence, confirming that artifacts did not generate these cDNAs. By combining the nucleotide sequence identified by these experiments, we determined the entire Magi 4 cDNA sequence composed of 529 nucleotides. As shown in Fig. 2, the open reading frame started with an initiation codon at nucleotide 89, which conforms to the Kozak rule [21], and terminated with a TAA stop codon at nucleotide 406. A polyadenylation consensus (ATTAAA) was found at nucleotide 509.

The open reading frame region was shown to encode a 105 residue Magi 4 precursor polypeptide. The Magi 4 transcript was predicted to be translated with Met[1], given that the hydrophobic moiety typical for a signal peptide region follows this Met residue. A sequence absolutely identical to CGSKRAWCKEKKDCCCGYNCVYA, determined by amino acid sequence analysis, was found at residues 63–85, followed by the 20 residues WYNQQSSCERKWKYLFTGEC terminated with a stop codon TAA (Fig. 2) and containing the predicted amino acid sequence YLFTGEC obtained by enzymatic cleavage and Edman degradation. The location of typical precursor convertase (PC) cleavage motifs RR or RXRR [22] at residues 59–62 supported the notion that the Magi 4 precursor is subjected to post-translational endoproteolysis at this site and that the Cys^{63} in the precursor is positioned at the N-terminus of the mature Magi 4. This finding was compatible with the fact that the first residue in native Magi 4 was shown to be a cysteine residue by amino acid sequence analysis. Taken together, these results revealed that the entire Magi 4 sequence was CGSKRAWCKEKKDCCCGYNCVYAWYNQQSSCERKWKYLFTGEC (Fig. 2). Furthermore, the presence of eight cysteine residues in the

Table 1
Amino acid sequences and molecular masses of *M. gigas* peptides (A) and identity and alignment to known spider toxins (B)

A

Peptide	Sequence	Calc. (Da)	Theo. (Da)
	1--------10--------20--------30--------40		
Magi 1	CMGYDIHCTDRLPCCFGLECVKTSGYWWYKKTYCRRKS*	4,563.0	4,564.3
Magi 2	CMGYDIECNENLPCCKHRKLECVETSGYWWYKRKYCRPIK*	4,940.3	4,941.8
Magi 3	GGCIKWNHSCQTTTLKCCGKCVVCYCHTPWGTNCRCDRTRLFCTED	5,222.8	5,223.1
Magi 4	CGSKRAWCKEKKDCCCGYNCVYAWYNQQSSCERKWKYLFTGEC	5,150.0	5,149.9
Magi 5	GCKLTFWKCKNKKECCGWNACALGICMPR	3,286.8	3,287.0
Magi 6	KCVDGSCDPYSSNAPRCCGSQICQCIFFVPCYCKYR*	4,036.4	4,037.6

B

Peptide	Sequence	Identity (%)	Reference
Magi 1	CMGYDIHCTDRLPCCFG--LECVKTSGYWWYKKTYCRRKS	-	This work
Magi 2	CMGYDIECNENLPCCKHRKLECVETSGYWWYKRKYCRPIK	68	This work
	******.*.:.**** ****:********:.*** .		
Magi 3	-GGCIKWNHSCQTTTLKCCGKCVVCYCHTPWGTNCRCDRTRLF--CTED	-	This work
PlTx-II	-ADCSATGDTCDHT-KKCCDDCYTCRCGTPWGANCRCDYYKAR--C-DT	43	[23]
PlTx-XII	AVKCIGWQETCNGN-LPCCNECVMCECN-IMGQNCRCNHPKATNEC-E-	35	[24]
	* .:*: . **..* * * * ****: : * :		
Magi 4	CGSKRAWCKEKKDCCCGYNCVYAWYNQQSSCERKWKYLFTGEC	-	This work
Versutoxin	CAKKRNWCGKTEDCCCPMKCVYAWYNEQGSCQSTISALWK-KC	47	[5]
Robustoxin	CAKKRNWCGKNEDCCCPMKCIYAWYNQQGSCQTTITGLFK-KC	50	[4]
	*..** ** :.:**** :*:*****:*.**: . . *:. :*		

Percentage identity is shown based on Magi 1, Magi 3 and Magi 4, respectively. The sequence alignment was obtained from http://www.ch.embnet.org/software/ClustalW.html. Gaps (-) have been introduced to enhance similarities.

deduced Magi 4 sequence suggested that this peptide should have four intramolecular disulfide bridges. The theoretical molecular mass value of the folded form of Magi 4 (with four disulfide bonds) was calculated to be 5150.0 Da, which was in good agreement with the observed value of the native form of 5149.9 Da. These results further confirmed the full-length sequence of Magi 4 as elucidated above.

3.3. *Similarities of* M. gigas *peptides to other spider toxins*

Magi 1, 2, 3, 4, 5 and 6 are composed of 38, 40, 46, 43, 29, and 38 amino acid residues, respectively (Table 1A). The amino acid sequences of Magi 3 and 4 share some similarities to the sequences of the neuropeptide PlTx-II from the venom of the American plectreurid spider *Plectreurys tristis* [23,24] and the δ-atracotoxins from the Australian hexathelid spiders *A. robustus* [4] and *H. versuta* [5] (Table 1B). The amino acid sequences of Magi 1 and 2 are homologous to each other, whereas both of them displayed no significant sequence similarity to any other peptides. The amino acid sequences Magi 5 and 6 share no similarities with amino acid sequences of known spider toxins.

3.4. *Biological activity and binding experiments to sodium channels*

The six pure peptide toxins were injected to *S. litura* lepidoptera larvae and to mice (C57/Bl6). Magi 1 did not cause any effect when injected to either mice (20 pmol/g) or insects (32.8 nmol/g). In insects, Magi 3 and 5 (10.3 and 8.6 nmol/g, respectively) produced paralysis to the lepidopteran larvae. However, paralysis inflicted by Magi 3 and Magi 5 was only temporary and the insects recovered after 2 h post injection. Magi 2, 4 and 6 induced flaccid paralysis to the larvae. The effects caused by those peptides were lethal with LD_{50}s of 17.6, 1.2 and 3.1 nmol/g, respectively. In mice, Magi 2 and 3 did not cause toxicity at 20 pmol/g. The three more hydrophobic toxins, Magi 4, 5, and 6, were lethal to mice with LD_{50}s of 0.15, 0.73 and 0.74 pmol/g, respectively (Table 2). The toxic symptoms produced by the intracranial injection of each hydrophobic peptide differ from the symptoms caused by the others. Injection of Magi 4 to mice produced instantaneous sweating, urination, salivation, agitation, convulsion and finally death. A low injection dose of Magi 5 caused lacrimation, closure of the eyes and sweating. At high injection dose of Magi 5 the mice showed extensive lacrimation and death. Injection of Magi 6 into mice caused awkwardness of movement, and respiration was labored until death. The flaccid paralysis caused by *M. gigas* peptides to larvae has also been observed after injection of scorpion depressant toxins to blow fly larvae. The scorpion depressant toxins affect sodium channel of insects [25,26]. Although the symptoms caused by injection of the pure Magi 4, 5, or 6 toxins to mice could not be directly linked to a particular ion channel receptor, they seem to act on the central nervous system [27]. To examine the mode of action of these peptide toxins in the nervous system, rat and cockroach brain synaptosomes were prepared. Competitive binding assays were performed using ^{125}I-labelled scorpion (CssIV, LqhαIT, Lqh2, BjxtrIT) and cone snail (δ-TxVIA) peptide toxins that bind to three different extracellular receptor sites of the sodium ion channel (Table 2). The ^{125}I-labelled scorpion toxins LqhαIT [28] and BjxtrIT [29] were used as probes for binding to site 3 and site 4 of the insect sodium channel, respectively [13]. The ^{125}I-labelled scorpion toxins Lqh2 [30] and CssIV [31] were used as probes for binding to site 3 and site 4 of the rat brain sodium channel, respectively [32,33]. Furthermore, the cone snail toxin, δ-conotoxin TxVIA, binds with high affinity to site 6 on sodium channels in rat central nervous system [19]. Magi 1, 2, 3, 4 and 5 were shown to inhibit the binding of the ^{125}I-labelled scorpion toxin LqhαIT to site 3 of the sodium channel of cockroach synaptosomes. Magi 4 showed the strongest competition against LqhαIT for site 3 of insect sodium channel with a K_i of 0.05 nM. Magi 2 and Magi 5 elicited the second and third most potent binding affinity to this site with K_is of 21 and 267 nM, respectively (Table 2). Magi 1 and 3 showed the weakest competition for the same site of insect sodium channels with K_is of 1747 and 1718 nM, respectively (Table 2, Fig. 3A). None of the six peptide toxins compete either with the scorpion toxin BjxtrIT (specific for site 4) or with the cone snail toxin δ-TxVIA (specific for site 6) in binding to cockroach synaptosomes (Table 2). In rat brain synaptosomes, only Magi 5 competed against the scorpion toxin CssIV in binding to site 4 with a K_i of 1.2 nM. Finally, none of the six peptide toxins competed either with the scorpion toxin Lqh2 (specific for site 3) or with the cone snail

Table 2
Biological activity and inhibitory dissociation constants (K_i) of *M. gigas* peptide toxins

Peptide	Insect ED_{50} [LD_{50}][a] (nmol/g)	Mouse LD_{50}[b] (pmol/g)	BjxtrIT K_i (nM)[c] (site 4, insects)	CssIV K_i (nM)[c] (site 4, rat)	LqhαIT K_i (nM)[c] (site 3, insects)	Lqh2 K_i (nM)[c] (site 3, rat)	TxVIA K_i (nM)[c] (site 6, rat/insect)
Magi 1	> 32.8 [> 32.8]	> 20	> 1000	> 1000	1747 ± 220	> 1000	> 1000
Magi 2	17.6 [17.6]	> 20	> 1000	> 1000	21 ± 1.8	> 1000	> 1000
Magi 3	10.3 [> 32.8]	> 20	> 1000	> 1000	1718 ± 193	> 1000	> 1000
Magi 4	1.2 [1.2]	0.15	> 1000	> 1000	0.05 ± 0.004	> 1000	> 1000
Magi 5	8.6 [> 32.8]	0.73	> 1000	1.2 ± 0.2	267 ± 31	> 1000	> 1000
Magi 6	3.1 [3.1]	0.74	> 1000	> 1000	> 2000	> 1000	> 1000
Cold toxin[d]			0.45 ± 0.07	0.38 ± 0.08	0.19 ± 0.03	0.2 ± 0.06	2.5 ± 0.4[e]

[a]For insects, the carrier solution was dH_2O and were injected with a microsyringe. ED_{50} and LD_{50} values were based on 50% paralysis and 50% mortality of treated insects at 2 and 24 h post injection, respectively. ED_{50} and LD_{50} (in brackets) values were obtained by probit analysis of data from six groups of 15 *S. litura* larvae.
[b]The carrier solution was 5 μl of BSA (20 mg/ml in 0.9% NaCl) and mice were injected with a 10 μl microsyringe fitted with a glass capillary. Intracranial injection in 5 week old male mice (C57/Bl6, approx. 20 g). LD_{50} values were based on 50% mortality of treated insects at 24 h post injection and were obtained by probit analysis of data from six groups of five animals each.
[c]K_i values were determined from competition binding curves of the indicated toxin on cockroach and rat neuronal membranes. The values represent the mean ± S.E.M. of at least two independent experiments.
[d]Cold toxin values represent the K_i of the competitive binding curves against the radiolabeled toxins by the native peptide.
[e]Value obtained from [19].

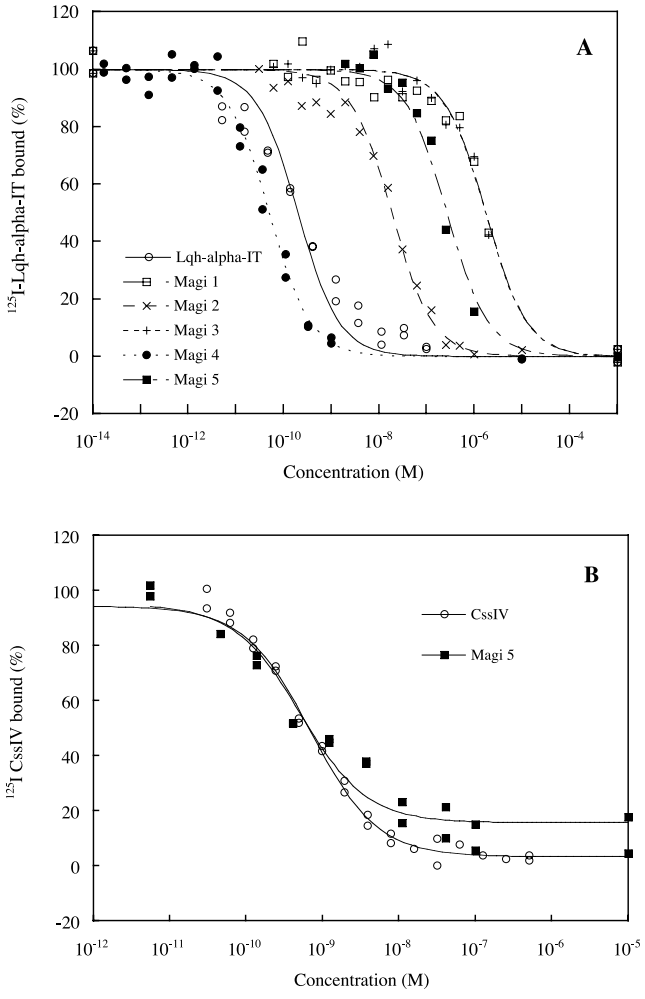

Fig. 3. Competitive experiments between radiolabeled scorpion toxins and cold *M. gigas* peptide toxins. A: Competition for [^{125}I]-LqhαIT binding by increasing concentrations of native scorpion LqhαIT and spider peptides Magi 1–5. Cockroach synaptosome protein concentration was 5 μg/ml. B: Competition for [^{125}I]CssIV binding by increasing concentrations of native scorpion CssIV and spider Magi 5. Non-specific binding was determined in the presence of 1 μM LqhαIT or 1 μM CssIV and subtracted from the data. Rat brain synaptosome protein concentration was 0.9 mg/ml. The values were fit with the non-linear Hill equation. The calculated K_i values are shown in Table 2.

toxin δ-TxVIA (site 6) in binding to rat brain synaptosomes (Table 2). Magi 6 could not compete with any radiolabeled toxins in binding to cockroach or rat brain sodium channels, and thus, the mode of action of Magi 6 remains unknown. However, the rapid and strong lethality of Magi 6 towards mice is suggestive of a strong antagonist action on the rat and insect nervous systems. Taken together, these results led to the conclusion that Magi 1–5 exhibit specific inhibitory effects on site 3 of the insect sodium channel and that Magi 5 also serves as the rat sodium channel blocker that specifically binds to site 4.

4. Discussion

In search of small molecular weight peptide ligands, with new pharmacological specificities, for the study of ionic channels, we investigated for the first time the peptide composition of the venom of the spider *M. gigas*. The mode of action of five of the major peptides of the venom of *M. gigas* was found to be towards the sodium channel.

The venom of *M. gigas* contains two toxic peptides (Magi 3 and 4) that have high amino acid sequence similarities to the peptide toxins found in the plectreurid North American spider *P. tristis*, and the toxic peptide toxins found in the hexathelid Australian spiders *A. robustus* and *H. versuta*. Although the hexathelids *M. gigas* and *A. robustus* as well as the plectreurid *P. tristis* belong to different suborders, peptides similar to Magi 3 and 4 have been conserved in these spiders. The amino acid sequences of the toxins Magi 1, 2, 5, and 6 from the venom of *M. gigas* are unique with no strong similarities to previously reported spider toxins.

Concerning the structural organization of the Magi 4 precursor, both sequences of full-length Magi 4 and of the precursor indicate that Magi 4 is matured through the post-translational cleavage at the N-terminus by PCs. Two processing mechanisms can be presumed. First, the twin arginine located immediately before the N-terminus of the Magi 4 sequence plays a role as an endoproteolytic site. Dibasic amino acids including the RR motif are the most prevalent endoproteolytic site in proprotein, prohormone, and proneuropeptides [22]. However, the Magi 4 sequence contains Lys^4-Arg^5, Lys^{11}-Lys^{12}, and Arg^{33}-Lys^{34} that can also be recognized by PCs as endoproteolytic sites, suggesting that PCs in the *M. gigas* venom are capable of cleaving the precursor exclusively at the di-arginine motif or that the tight folded form of Magi 4 owing to four disulfide bridges hinders the approach of PCs to any dibasic motifs in Magi 4 sequence. Alternatively, RVRR, instead of RR, is likely to serve as the cleavage site, which is supported by the fact that some PCs specialize in endoproteolysis at the RXRR motif [22]. Analysis of the cDNA libraries of other spiders such as *Atrax* sp. [34] and *P. tristis* [35] indicated that ω-atracotoxins and PlTx toxins are initially synthesized as prepro-forms which undergo elimination of their signal peptide region followed by additional processing at their N- and/or C-termini to produce the mature products. ω-Atracotoxin-Hi2a and PlTx-VIII toxin are flanked at their N- and/or C-termini by endoproteolytic sites Glu-Arg/Gly-Arg [34] and Glu-Arg/Arg-Arg [35], respectively, whereas in Magi 4 the processing site at the N-terminus was Arg/Arg. These findings indicate that the post-translational processing of peptide toxin precursors is dependent on the species from which the peptide toxins were isolated.

The biological activity observed by direct injection of Magi 1–5 toxins in lepidoptera correlates well with the competitive binding experiments. Even though Magi 1 competitively binds to site 3 against the scorpion toxin LqhαIT, it did not cause paralytic effects to insects. The concentration of Magi 1 injected to the lepidoptera larvae could be not enough for causing paralysis to the larvae, or the binding of Magi 1 to insect Na^+ channels may be classified as 'silent binding' as seen in other peptide toxins [19]. Magi 3 and 5 were shown to cause only paralytic effects but no lethal effects to lepidoptera. Magi 3 and 5 also had the highest K_i in competitive binding to site 3 against the insect toxin LqhαIT. Magi 2 and 4 caused lethal toxicity to lepidopteran larvae in good correlation to the competitive binding data (Fig. 1A). In mice, only Magi 5 was found to be lethal and compete with the sodium channel toxin CssIV. Magi 4 and 6 produced neurotoxic effects on mice; however, they were unable to compete with any of the three

radiolabeled toxins in binding to site 3, site 4 and site 6 of the sodium channel. Therefore, Magi 4 and 6 seem to target different binding site in the sodium channel of rat brain synaptosomes or to target different cellular receptors in mice.

Magi 1 and 2 share 68% amino acid sequence homology with each other since 26 of their 38 amino acids are identical. Despite high amino acid identity between these homologous toxins, Magi 2 exhibited 80-fold more potent binding activity to site 3 of the insect sodium channel than Magi 1. The most remarkable amino acid differences between these two peptides are Phe^{16}-Gly^{17} for Magi 1 and Lys^{16}–Lys^{19} for Magi 2 where Magi 2 has a cluster of cationic amino acids and a guanidinium group at Arg^{18} (Table 1). A positive surface charge has been implicated for recognition of sodium channel receptors by scorpion toxins [13,15], and a guanidinium group is responsible for blocking sodium ion channels by cone snail toxins [14,36]. Therefore, this amino acid segment found in Magi 2 but not in Magi 1 most probably accounts for this disparity for receptor recognition in cockroach synaptosomes, and these amino acids exclusively present in Magi 2 are expected to confer such high affinity with the low K_i value to site 3 of the cockroach sodium channel. Since Magi 2 had no effect in mice, this peptide seems highly specific for insects. Magi 2, like the sea anemone toxin ATX-II [37], are the only peptides reported having three disulfide bridges that bind to site 3 of the sodium channel. These findings are in contrast with the fact that all scorpion and spider toxins that bind to site 3 of the sodium channel contain four disulfide bridges.

Magi 3 and the peptide toxin PlTx-II from the plectreurid spider *P. tristis* share 43% identity in their amino acid sequences. PlTx-II has been reported to be a calcium ion channel blocker in insects [38]. Moreover, Magi 3 was shown to bind to the insect sodium channel (Fig. 3 and Table 2). In combination, these findings indicate the possibility that Magi 3 also recognizes insect calcium channels. Some spider toxins have shown promiscuity across ion channel families. For example, ProTx-I, from the venom of the theraphosid *T. pruriens*, inhibits the tetrodotoxin-resistant Na channel, Nav 1.8, and also modifies the currents of the Cav 3.1 (α1G, T-type) and the Kv 2.1 channels [8]. Moreover, grammotoxin-SIA inhibits not only several types of calcium channels (N-type, P/Q-type) but also potassium channels (*drk*1 K^+) [12,39].

Magi 4 was able to compete with the insect toxin LqhαIT at subpicomolar concentrations in binding to site 3. Similar toxins such as versutoxin and atracotoxin also competitively bind to insect synaptosomes against the insect toxin LqhαIT at picomolar concentrations [6,40]. On the other hand, Magi 4 was found to fail to interact with site 3 of the sodium channel in rat synaptosomes, given that Magi 4, unlike δ-atracotoxin [6], showed no competitive binding activity against the rat sodium channel site 3 ligand, Lqh2. In addition, a newly isolated toxin, δ-atracotoxin-HV1b, shows a marked contrast with Magi 4 because it completely lacks insecticidal activity but displays an inhibitory effect on the rat sodium channel [41]. Therefore, Magi 4 and δ-atracotoxins illustrate variability for sodium channel recognition in rat and insect neurons. Magi 4 is lethal to mice by intracranial injection, suggesting that Magi 4 could interact with the mammalian sodium channel at a non-identical site to that of Lqh2.

Magi 5 showed displacement from 80–90% of the total binding of the β-scorpion toxin CssIV that binds to site 4 of the rat brain sodium channel. Antibodies specific for the rat brain sodium channel subtypes rI and rII have shown that the content of sodium channels purified from rat brain is approximately 18% rI and 80% rII [42]. In this context, Magi 5 displaced partially the binding of the β-scorpion toxin, suggesting that Magi 5 may recognize a subpopulation of brain sodium channels. This particularity of Magi 5 needs further investigations to be confirmed. Moreover, Magi 5 competed with the insect sodium toxin LqhαIT in binding to site 3 of sodium channels, suggesting that both site 3 of insect sodium channels and site 4 of the rat brain sodium channel might embrace similar molecular surfaces. Magi 4 and 5 showed strong competition for the binding to site 3 and site 4 of insect and rat brain sodium channels, respectively. Some questions could arise from their competitive binding activities. First, why does Magi 4 bind to site 3 in insect, but fail to recognize (even being lethal to mice) the related site 3 in rat brain synaptosomes? And second, why can Magi 5 bind to both site 3 in insects and site 4 in mammals? It is likely that binding sites in sodium channels are not independent structural sites but belong to a common macrosite or 'hot spot' as has been proposed [43], where small molecular weight toxins (i.e. spider toxins) could be more easily accommodated than large molecular weight toxins (i.e. scorpion toxins). Spider toxins, especially those with low molecular masses, are becoming valuable tools for the investigation of the topology of ion channels. These low molecular weight ion channel modifiers could be synthesized chemically and then mutated for studies towards the molecular recognition of its receptors that could permit the engineering of novel synthetic drugs. Molecular recognition of toxin for its receptors could be achieved also by ion channel mutagenesis. Studies on the molecular interaction between animal toxins and ion channel receptors have been increasingly reported [9,11,12]. Magi 1, 2, 3, 4 and 5 are short molecular toxins that can be easily synthesized chemically for further exploration of ion channels in both insects and mammals. Magi 6 is also a short molecular toxin, although its mode of action remains unknown.

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