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SEQUENCE COMPARISON AND COMPUTER MODELLING OF CARDIOTOXINS AND COBROTOXIN ISOLATED FROM TAIWAN COBRA

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Six cardiotoxins and one neurotoxin isolated and purified from the Taiwan cobra venom (*Naja naja atra*) possess distinct pharmacological and biochemical properties despite the existence of a grossly similar tertiary structure among these toxins, *i. e.*, a core consisting of a series of short loops and four disulfide bridges. A systematic structure comparison of these major toxin isoforms was made by the secondary-structure predictions together with computer model-building based on the primary sequences and the established X-ray and NMR structures of one published cardiotoxin isoform and cobrotoxin. It is of interest to find that some defined and subtle differences can be detected upon the superposition of these three-dimensional polypeptide chains, which may reflect the intrinsic differences in the surface hydrophobicity of cardiotoxins and cobrotoxin as revealed by hydropathy profiles of these toxins in one of three major loops. The differences seem to correlate with different inhibitory activities exhibited by cardiotoxins in contrast to the lack of activity by cobrotoxin on protein kinase C (PKC).

There have been many reports on the characterization of varied types of toxins from the *Elapidae* family of snakes, notably in the cobras of *Naja* genus [1-3]. Among these toxins cardiotoxins, neurotoxins and phospholipases A_2 are the three major classes of polypeptides involved in the toxicity and pharmacology of snake bites caused by these snakes. All these toxin molecules are chemically and thermally very stable. In contrast to another prominent group of structurally similar neurotoxins (α-cobratoxins) from elapid species with well-established acetylcholine receptors and modes of action at the molecular level [4], cardiotoxins showed no defined cellular targets and very diverse pharmacological functions. There are currently more than 60 sequences of cardiotoxins determined from varied sources of snake families [2,3]. When many homologous sequences are known for a particular class of proteins, comparative analysis of the data can prove to be most informative.

Recently we reported that cobra cardiotoxin and its various isoforms [5], like other naturally occurring bioactive amphiphilic polypeptides such as mastoparan [6] and melittin [7], are specific and strong inhibitors of protein kinase C (PKC) [8]. Interestingly receptor-specific α-cobratoxin (cobrotoxin) of the same cobra venom, in contrast, showed no effect on PKC. In light of the link between PKC inhibition and CTXs regarding implications for the actual cellular

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target of these small toxin peptides, it is deemed essential to isolate and characterize natural cardiotoxin isoforms or variants in order to study their differential effects on this important signal-transduction pathway. In a preliminary report we have achieved the determination of the primary sequence of one novel cardiotoxin distinct from the four major CTXs reported previously from Taiwan cobra [9]. In order to gain some insight into the structural difference present in these closely-related analogous toxins, a systematic structure comparison of these cardiotoxin isoforms and one functionally distinct cobrotoxin was made by the secondary-structure predictions together with computer model-building based on their primary sequences and the established X-ray [10] and NMR [11] structures of one related cardiotoxin and cobrotoxin. In the present study the minor though defined structural differences were found in these CTX analogues, and a more profound difference between CTXs and cobrotoxin was also revealed by the superposition of these three-dimensional structures by computer graphics.

MATERIALS AND METHODS

Purification and sequence analysis of venom toxins

The lyophilized venom powder was obtained from Sigma Chemical Company (St. Louis, MO). Various toxins were isolated by cation-exchange chromatography on an open column (50 x 2.5 cm I.D.) packed with TSK CM-650 (M) as described before [5]. Dissolved venom powder in 0.025 M ammonium acetate, pH 6.0 starting buffer (20-50 mg/ml) was applied to TSK CM-650 column equilibrated with the same buffer. More than 5 cardiotoxin fractions were lastly eluted in a gradient of 0.7-1.0 M ammonium acetate, pH 5.9 buffer [9]. A reversed-phase HPLC (RP-HPLC) was also carried out on a Hitachi's liquid chromatograph with a model L-6200 pump and a variable UV monitor. The column (4.6 x 250 mm, Vydac RP-C_8, 5.0 μm bead) was used to purify and desalt the dried toxin fractions isolated from the above ion-exchange chromatographies. The purities of the isolated toxins were checked by SDS-polyacrylamide slab gel (5 % stacking/ 16 % resolving gel) as described before [12].

The complete sequences of the purified CTX fractions from reversed-phase HPLC column were carried out by automated Edman degradation with a pulsed-liquid phase protein sequencer (Model 477A, Applied Biosystems, Foster City, CA, USA). For some highly purified CTXs the complete 60 residues can be determined in a single non-stop run of successive Edman degradation. By comparing the amino-acid compositions and primary sequences determined with the sequencer the unambiguous sequence assignment of each CTX can be made. These CTX sequences and that of cobrotoxin [13] were used for comparison of structural homology among these toxins based on established X-ray or NMR structures [10,11].

Hydropathy profile and secondary structure prediction

A program analysis using Macintosh computers of the surface hydrophobicity of six CTXs and cobrotoxin along the loop II segment (#Cys21-#Cys42 according to the residue numbering in CTX) based on the Kyte-Doolittle hydropathy scale [14], was carried out on the MacVector sequence analysis software (International Biotechnologies, Inc., New Haven, CT). The signs of the values have been reversed in order to plot the hydrophilicity instead of hydrophobicity profile. A window of size N=7 was run along the length of toxin segments; for each window, the hydropathy values of the 7 amino acids were summed and divided by 7 to obtain the average hydrophilicity per residue for the window. Values above the axis denote hydrophilic regions which may be exposed on the outside of the protein molecule whereas those values below the axis indicate hydrophobic regions which tend to be buried inside the protein. For the prediction of the secondary structures for these CTXs, the prediction methods of Chou and Fasman [15] and Garnier *et al.* [16] together with a consensus joint prediction of two methods were used .

Molecular model building

The sequences of the five cardiotoxins (CTX I-IV and CTXn) [9] were aligned to locate sequence-conserved regions. The coordinates of one homologous cardiotoxin (from *Naja m. mossambica*) [10] which was previously determined by X-ray diffraction analysis and refined to 2.5 Å [17] were used as a template for model building of cardiotoxins by employing the graphics program Insight II (Biosym, 1992) on a Silicon Graphics IRIS 4D/35 workstation.

Under Homology module of Insight II environment, multiple sequence alignment algorithm was used to align five cardiotoxin sequences to the sequence of cardiotoxin from *Naja m. mossambica*. The main-chain coordinates of the modelled cardiotoxins were copied from existing cardiotoxin structure. The coordinates of the side-chain and the undefined terminal residues were built and relaxed by using "End Repair" and "Relax" function in Homology module, which both use the Discover program with "CVFF" forcefield to remove close contacts and reduce strains. The complete structures were further minimized by heating up to 300 °K, equilibration for 0.5 ps (500 steps), 200 steps of steepest gradient minimization and 2000 steps of conjugate gradient minimization to optimize the hydrogen bonds, ion pair and hydrophobic interactions. For the comparison of one of the modelled CTX structures with the solution structure of cobrotoxin determined from NMR [11], we first input the sequence of cobrotoxin [13] into the multiple sequence alignment algorithm to obtain a modelled cobrotoxin structure based on X-ray diffraction coordinates of CTX [17], then compare this structure with that of cobrotoxin using the coordinates determined from NMR. In general the average RMS (root-mean-square) deviation between the two is less than 3 Å for most of the backbone atoms of these polypeptide chains.

RESULTS AND DISCUSSION

Cardiotoxins were originally named for their *in vivo* action on the heart which results in cardiac arrhythmia, but they have also been shown to have cytotoxic activity on all types of excitable and non-excitable cells (therefore also called cytotoxins). They are a group of toxic polypeptides of about 60-63 amino-acid residues present abundantly in the elapid family of snakes, comprising about 45-55 % total proteins in the crude venom of Taiwan cobra [1]. In the previous study [9] we have applied multiple-step chromatographies with improved efficiency of separation for the isolation and characterization of several cardiotoxins from the Taiwan cobra. Since this group of toxins showed very similar polypeptide folding pattern yet exhibited distinctly different pharmacological properties from another group of neurotoxins present in the same venom, it is deemed essential to find minor and defined structural or conformational differences between the two in order to account for these functional differences at the molecular level. Currently the structural comparison based on X-ray and/or NMR data of structurally homologous proteins coupled with state-of-the-art computer-graphic modelling is increasingly becoming popular among protein chemists. There is much additional insights to be gained by the detailed structural comparison of these sequence-related toxin analogues in order to provide a means of correlating structure/function properties.

Purification and sequence analysis of venom toxins from the Taiwan cobra, Naja naja atra

Six cardiotoxins designated as CTX I to CTX V plus one novel cardiotoxin (CTXn) were identified from the improved elution separation of the crude venom on TSK CM-650 cation-exchange column [9]. **Table 1** shows the physicochemical parameters of these CTXs and one major α-cobratoxin, *i.e.* cobrotoxin named for the neurotoxin isolated from the Taiwan cobra [13]. We have demonstrated that numerous pure cardiotoxin isoforms of snake venoms from *Elapidae* can be resolved by the use of RP-HPLC and their differences in respective retention times seem to correlate with their surface hydrophobicity (Hφ) since all chromatographic media other than reversed-phase HPLC cannot resolve clearly all these toxins with closely-similar molecular weights (Mr) and isoelectric points (pI). It is to be emphasized that CTX V (C5) identified here is not the basic cardiotoxin analogue with membrane-fusion effect, as reported

Table 1. Hydrophobicities, HPLC retention times, isoelectric points and molecular weights of purified cardiotoxins and cobrotoxin isolated from *Naja naja atra*

	CTX I	CTX II	CTX III	CTX IV	CTX V	CTXn	NTX
Hϕ	0.12	0.11	0.14	-0.03	0.09	0.16	-1.27
R.T. (min)	16.356	16.000	16.735	15.255	15.971	16.251	7.983
pI	10.039	10.129	10.181	10.313	10.053	10.129	9.741
Mr	6700	6750	6747	6793	7013	6688	6956

Hϕ is the hydrophobicity index as defined in Ref. [14]. R.T. are retention times (in min) for each CTX and cobrotoxin (NTX) as determined from reversed-phase HPLC of each purified toxin. pI and Mr denote isoelectric points and molecular weights calculated from the primary sequences of toxins.

recently by Chien et al. [18]. In contrast with their characterization of Taiwan cobra venom, it was of surprise to find that we could not detect such a component in all six fractions of CTXs from the crude venom of same cobra species by cation-exchange chromatography. Nevertheless we did find one new cardiotoxin heretofore never described in the venom of *Naja naja atra*, *i.e.* CTXn fraction. A systematic comparison of the primary, secondary and tertiary structures based on the determined sequences, secondary-structure prediction and computer graphics was hence carried out with the aim of locating some subtle differences to account for their functional diversity.

Structural comparison, hydropathy profile and secondary structure prediction

In the pair-wise sequence analysis of the amino-acid sequences for various CTXs and cobrotoxin (**Fig. 1**), it is found that these 6 CTXs show about 80-93 % sequence identity to one another, *i.e.* 80 %, 82 %, 88 %, 90 % and 93 % sequence identity exists for the pairs CTX I/CTXn, CTX I/CTX II, CTX III/CTX IV, CTX II/CTX III and CTX II/CTX V respectively. In contrast only about 25-30 % sequence similarity is found between various CTXs and cobrotoxin. All these toxins possess identical arrangement of four disulfide bonds with similar intercystine loop lengths along the whole polypeptide chain. This certainly underlies the closer tertiary structures observed in the three-dimensional graphics of these two classes of toxins (*vide infra*). The structural homology between neurotoxins and cardiotoxins is essentially due to the similar location of the four disulfide bridges. This sequence homology contrasts with the completely different modes of action of the two types of toxins as reported in the literature [1-3].

All these determined sequences have laid a firm molecular basis for a preliminary secondary-structure prediction together with the construction of tertiary structures based on the coordinates obtained from the X-ray and NMR analysis of one homologous cardiotoxin [10,17] and cobrotoxin [11] respectively. We have compared the general distribution of surface-charge groups in these structurally related toxins using the program analysis of surface hydrophilicity along one of the segments (residues #21-42) corresponding to the loop II in the

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          10        20        30        40        50        60
LKCNKLIPIASKTCPAGKNLCYKMFMMSDLTIPVKRGCIDVCPKSNLLVKYVCCNTDRCN      CTX-I (C1)

LKCNKLVPLFYKTCPAGKNLCYKMFMVSNLTVPVKRGCIDVCPKNSALVKYVCCNTDRCN      CTXII (C2)

LKCNKLVPLFYKTCPAGKNLCYKMFMVATPKVPVKRGCIDVCPKSSLLVKYVCCNTDRCN      CTXIII (C3)

RKCNKLVPLFYKTCPAGKNLCYKMFMVSNLTVPVKRGCIDVCPKNSALVKYVCCNTDRCN      CTXIV (C4)

LKCNKLVPLFYKTCPAGKNLCYKMFMVSNKMVPVKRGCIDVCPKSSLLVKYVCCNTDRCN      CTXV (C5)

LKCNQLIPPFYKTCAAGKNLCYKMFMVAAPKVPVKRGCIDVCPKSSLLVKYVCCNTDRCN      CTXn

LECHNQQSSQTPTTTGCSGGETNCYKKRWRDHRGYRTERGCGCPSVKNGIEINCCTTDRCNN   Cobrotoxin
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Fig. 1. Comparison of the sequences of six cardiotoxin isoforms and cobrotoxin from *Naja naja atra*. All CTX sequences listed were taken from the previous studies [9,13]. CTXn is the novel cardiotoxin first reported from the Taiwan cobra. The amino-acid residues #21-#42 of CTXs and #24-#43 of cobrotoxin corresponding to the loop II segments in the tertiary structures are underlined to indicate the close amino-acid similarity among CTXs and distinct differences between CTXs and cobrotoxin in this major flexible loop. The eight cysteine residues situated at similar locations along the polypeptide chains are highlighted in bold letters. Amino acid residues are denoted by one-letter symbols.

X-ray structure of one homologous CTX [10] based on the Kyte-Doolittle hydropathy scale [14]. The hydrophobicity indices Hϕ for each cardiotoxin and cobrotoxin based on this scale were also caiculated and listed in Table 1. **Fig. 2** shows the hydropathy profiles and predicted secondary-structures for this major central loop present in CTXs and cobrotoxin. It is noteworthy that all five CTXs except CTXn and CTX I are devoid of α-helical structures based on a consensus joint prediction of two prediction methods [15,16]. The dominance of an anti-parallel β-sheet secondary structure in CTXs is in general consistent with CD and Raman studies of these toxins [19,20]. The overall hydropathy profiles also indicate a great similarity for the predominant distribution of hydrophobic amino-acids along the loop-II segments of these CTX chains, in contrast to the presence of almost exclusively hydrophilic region in loop II of cobrotoxin. Apparently this loop II segment may fold differently in CTXs and neurotoxic cobrotoxin as judged by the distribution of hydrophobic amino acids.

Molecular model building and structural comparison of cardiotoxin isoforms and cobrotoxin

Fig. 3 illustrates the folding and arrangement of polypeptide chains in these CTX isoforms. The topology of these polypeptides is very similar to that found in short and long snake neurotoxins [3,11], which block the nicotinic acetylcholine receptor. The amino acid sequence of CTX from *Naja m. mossambica* (PDB code: 1CDT) [10,17] used as the basis reference set for modelling is highly similar to CTXs (identity is about 74-80 %). Therefore it is to be expected that the tertiary structures of these CTXs will be similar to the previous crystal structure of 1CDT except the loop regions. The structures of loops are usually very difficult to

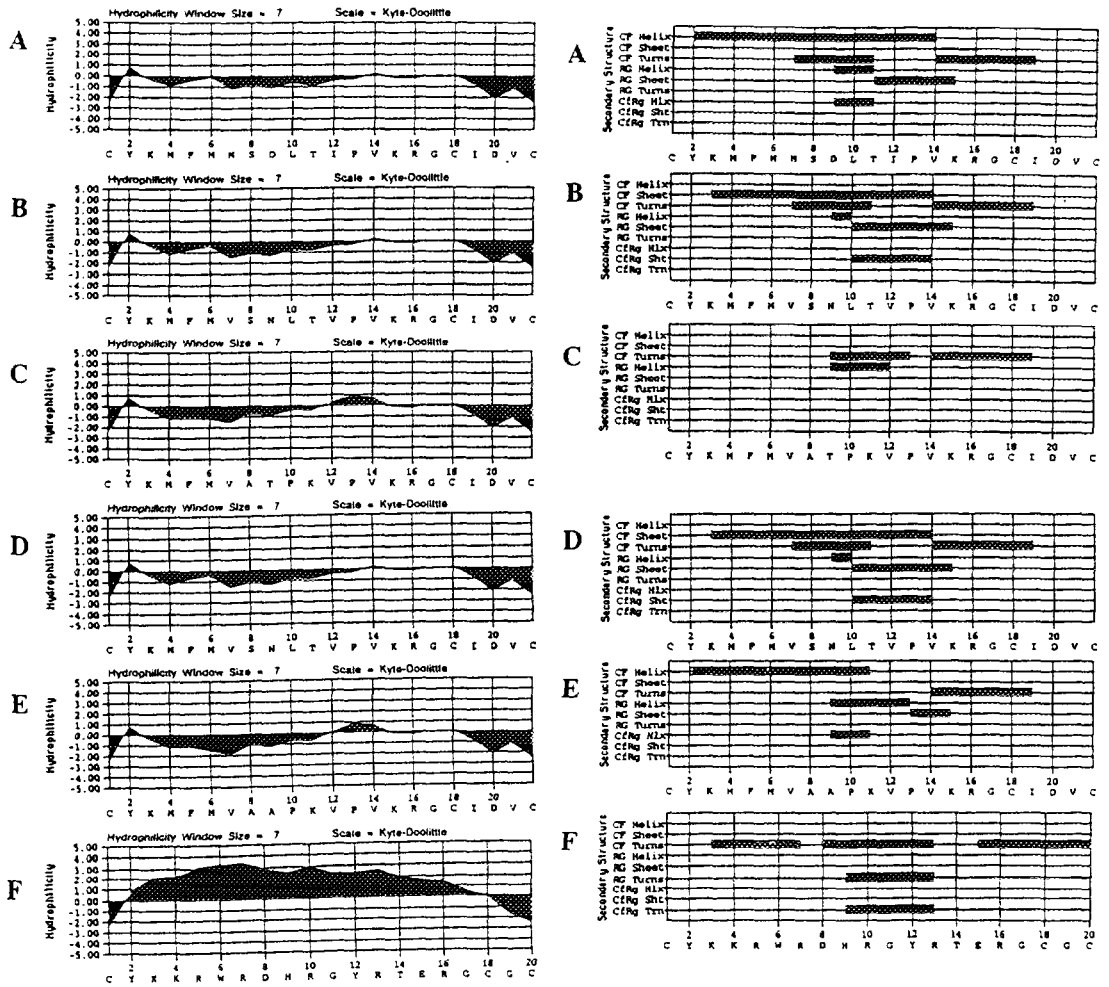
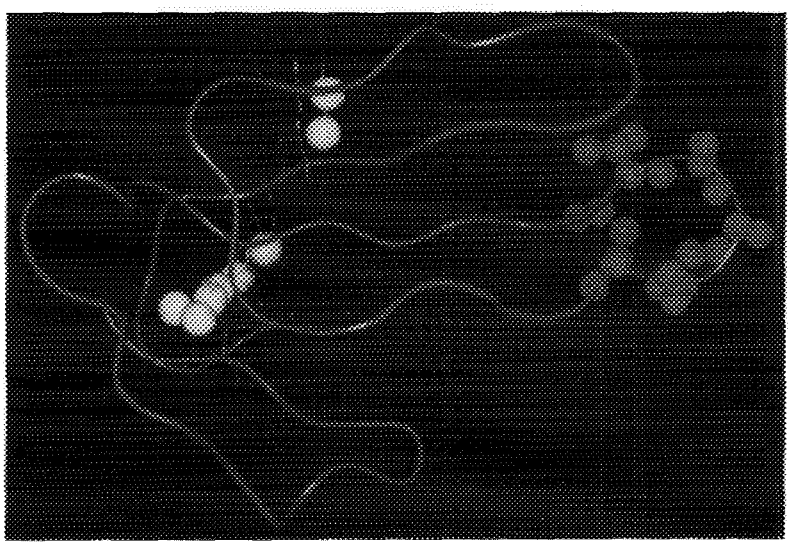
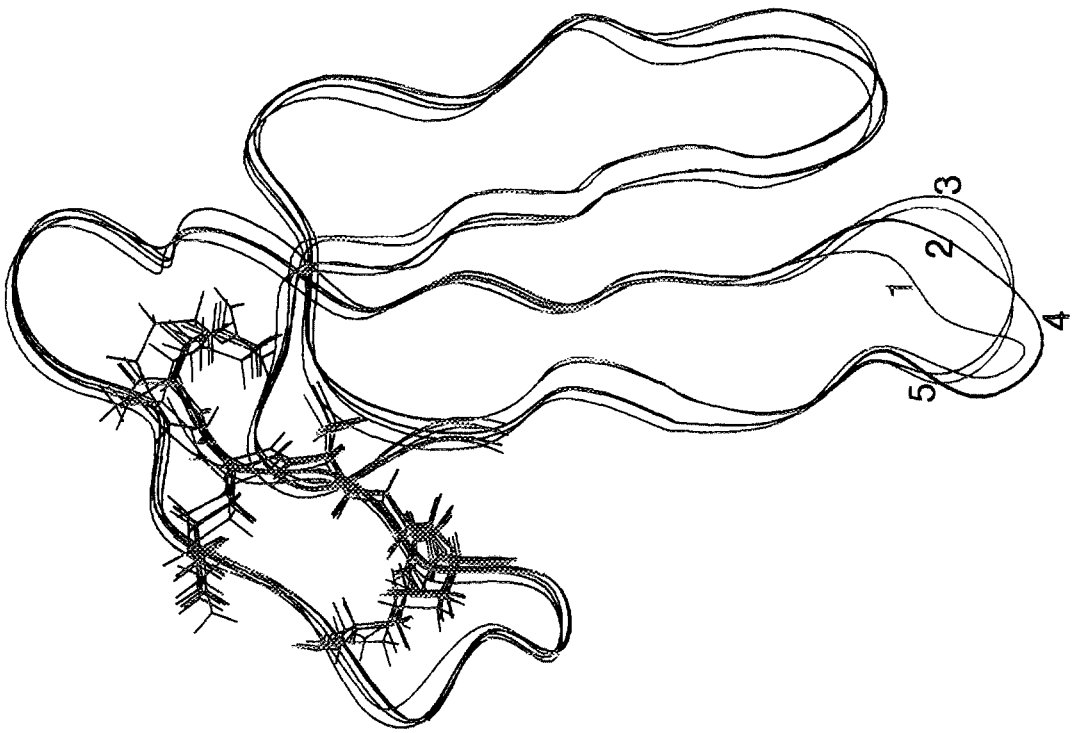


Fig. 2. Hydropathy profiles and secondary-structure predictions of cardiotoxin isoforms and cobrotoxin on the segments corresponding to the central flexible loop II. The analysis of the local hydrophilicity and conformational analysis of each structural segment (α-helix, β-sheet and β-turns) along the segments (residues #21-#42 for CTX I-IV (**A-D**) and CTXn (**E**), and residues #24-#43 of cobrotoxin (**F**)) were based on the methods of Kyte and Doolittle [14], Chou and Fasman (CF) [15] and Garnier *et al.* (RG) [16] and a consensus joint result (CfRg). The sequences are listed below each graph for easy comparison. It is noteworthy that CTXs show similar hydrophobic profiles in this loop whereas that of cobrotoxin mainly hydrophilic.

be predicted precisely from the primary sequence alone, except the special cases where the structural motifs are well defined such as "canonical structures" present in some proteins. Major differences occur in the conformation of the central loop (loop II, residues #21-42), resulting in a change in the cavity of the molecule. Careful scrutiny of these superimposed CTX models indicated that the major conformational differences among these CTXs seem to lie in the segment of residues #27-31 of loop II. The conformation of loop II is different from loop I and III, and the RMS distance after superposition is larger and more variable based on the coordinates of the defined X-ray structure of 1CDT [17]. This is consistent with the

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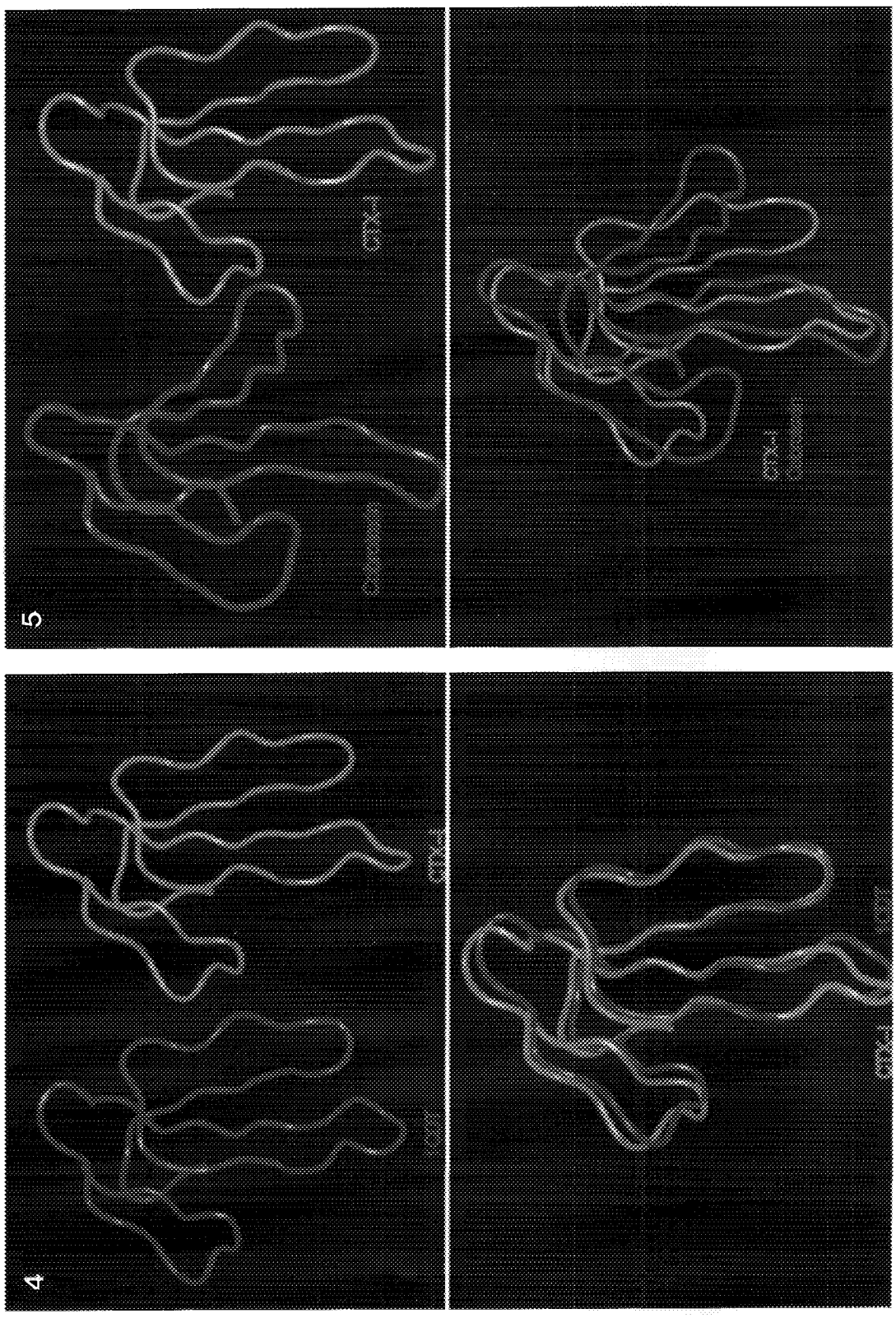
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sequence comparison in Fig. 2, which points out that the major sequence heterogeneity occurs at the segment of residues #26-31. Generally speaking, all these CTXs consist of a small core containing 4 disulfide bonds from which three adjacent loops (I, II and III) forming multiple-stranded antiparallel β-sheet protrude outwards (inset of **Fig. 3**). We have made a superposition of the modelled, coiled-structure of CTX-1 onto that of the reference X-ray structure of CTX, *i.e.* 1CDT (**Fig. 4**). It is clearly seen that the general overall fit between two structures is good except that the central loop II seems to exhibit a more variable and flexible mobility relative to the mean-static structure of 1CDT at the external loop-tip region.

As regards the tertiary-structure homology between various neurotoxins and cardiotoxins, it could well arise from the 8 critically important cysteines situated in similar locations to form a hydrophobic core consolidated by the formation of four disulfide bridges. The comparison leads to the same distinction between a stable core and more variable and flexible external loops in the graphic structures of both CTX-I and cobrotoxin (**Fig. 5**). Here the fit is somewhat acceptable for the common hydrophobic core formed at the top region of three loops in CTX-1 and cobrotoxin structures, yet the pronounced distinction in the topology of all three external loops certainly underlies the structural basis for the functional diversity between two classes of snake toxins. In the NMR study of CTX-III from the Taiwan cobra, Bhaskaran et al. [21] obtained a solution structure of CTX with the average value of atomic RMS deviation between 20 simulated annealing structures and their geometric mean of about 0.9 Å for the backbone atoms. The good agreement between the NMR structure of CTX-III and X-ray structure of 1CDT is clearly indicated except at segments of the turns present at the exposed loop/surface regions. Therefore our modelling study of various CTX isoforms based on X-ray structure of 1CDT (Fig. 3 & Fig. 4) is at least in good accord with both X-ray and NMR structures of CTX. On the other hand, for their comparison of NMR structures of CTX-III [21] and cobrotoxin [11], some larger differences especially in the loops I and II of these molecules (distinctly oriented side-chains of residues at these positions) were also identified. The

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Fig. 3. Superposition of the graphic ribbon models of cardiotoxins I-IV and CTXn with characteristic three-loop structures. From left to right are loop I (residue #1-17), loop II (central loop, residue #21-42) and loop III (residue #43-55), respectively. The N-terminal segment with five amino acids designated by wire-frame drawing is shown at the loop I. It is noteworthy that the tip region of the central loop (residue #27-31) shows greater differences among these five cardiotoxins than the other two loops, with CTX I-IV denoted by lines 1-4 and CTXn by line 5, respectively. [Inset] The computer graphics of the ribbon drawing of the novel cardiotoxin (CTXn). The amino acids are denoted by van der Waals ball representation, with those 8 cysteine residues at the base of the loops shown in yellow and the five amino-acid residues (#27-31, Val-Ala-Ala-Pro-Lys) at the tip of the central loop shown in green.

Fig. 4. Comparison and superposition of the graphic ribbon model of CTX-1 with that of one analogous CTX based on X-ray structure (PDB code: **1CDT**) [17]. The characteristic three-loop structures are shown for both toxins and the relative orientation of the loop structures is similar to that of Fig. 3. Note that the topological fit between these two models are excellent except that of central loop II region showing the average value of RMS deviation larger than the other two loops. Loop I region showed smaller flexibility than that of loop II.

Fig. 5. Comparison and superposition of the graphic ribbon model of CTX-1 with that of cobrotoxin. Both structures are modelled based on their primary sequences and the coordinates of X-ray structure of **1CDT** shown in Fig. 4. Note that the model depicted here for cobrotoxin is essentially similar to that of cobrotoxin based on NMR solution structure of this toxin. It is also noteworthy that these two structures cannot superimpose upon each other as that shown in Fig. 4 and the average RMS deviation between the two is smaller than 3 Å only for the backbone atoms of the hydrophobic core region at the top.

hydrophobic flat surface and distribution of hydrophobic residues along the above two loops are considered to be important sites that are expected to play a major role in the activity of CTX relative to other short neurotoxins such as cobrotoxin shown here. This observation appears to strengthen our previous supposition that distinct effects of CTX isoforms and cobrotoxin on the inhibition of PKC activity located on the membranes of various cells [5] seemed to correlate with different distributions of hydrophobic segments on the surface of these toxins. Therefore we conclude that the different effects of CTX isoforms reported on cell membranes [22,23] may lie in the subtle difference of surface hydrophobicity as exemplified on the flexible loop II region of these CTX analogues whereas the distinctly different biological effects exhibited by cobrotoxin and CTXs may reflect more extensive differences both at the primary and tertiary structures of three external surface loops.

CONCLUSION

The search for the structural basis to account for different functional properties exhibited by CTXs and cobrotoxin has prompted us to carry out secondary- and tertiary-structural comparisons based on closely related primary sequences and X-ray and/or NMR structures of these toxins. The target of neurotoxins was identified as the nicotinic acetylcholine receptor at the post-synaptic level of the neuromuscular junction [4]. Targets of surface-active CTXs are probably localized on various cell membranes, which CTXs can cause rapid membrane lysis or depolarization [1,3]. The previous study [5] revealed a more specific molecular interaction of CTX isoforms on protein kinase C (PKC), in great contrast to almost total lack of activity for cobrotoxin. A positive correlation seemed to exist between hydrophobicities (Hϕ) of multiple cardiotoxin isoforms and their corresponding inhibitory actions on PKC (unpublished results).

On the basis of these modelling studies, CTXs and cobrotoxin possess similar gross topology, *i. e.* a core consisting of a series of short loops and β-sheets and linked tightly by the four disulfide bridges. This core contrasts with three less well-ordered and more flexible longer loops constituted by several antiparallel β-sheet secondary structures, especially the protruding and rather floppy loop II. Therefore unusual functional differences between these toxins do not arise from any gross structural differences. The superpositions of constructed molecular models for various CTXs and cobrotoxin indeed detected salient RMS deviation on this central major loops among these CTX isoforms whereas a more explicit and pronounced structural variation along all three loops between CTX and cobrotoxin was found. In contrast to the conclusion reached by Rees et al. [17] suggesting that loop I rather than loop II plays an important role in the toxic action of CTX, our graphic modelling study coupled with NMR solution study of CTX III [21] revealed otherwise. Site-specific mutagenesis of the residues on loop I and loop II may be conducive to resolving this important aspect of structure/function correlation on various CTXs and their drastic functional difference with cobrotoxin.

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