Enzymatic Activity and Inhibition of the Neurotoxic Complex Vipoxin from the Venom of *Vipera ammodytes meridionalis*

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Neurotoxin, Phospholipase A2, Snake Venom

Vipoxin from the venom of *Vipera ammodytes meridionalis* is an unique neurotoxic complex between a toxic phospholipase A2 and a highly homologous non-toxic protein inhibitor. It is an example of evolution of a catalytic and toxic function into inhibitory and non-toxic one. The activity of the *V. ammodytes meridionalis* toxin is 1.7 times higher than that of the closely related (92% sequence identity) neurotoxic complex RV4/RV7 from the venom of *Vipera russelli formosensis*. The enhanced enzymatic activity of vipoxin is attributed to limited structural changes, in particular to the substitutions G54R and Q78K in the PLA2 subunit of the complex and to the T54R substitution in the inhibitor.
Oleyloxyethylphosphocholine, aristolochic acid and vitamin E suppressed the enzymatic activity of vipoxin and its isolated PLA2 subunit. These compounds influence inflammatory processes in which PLA2 is implicated. The peptide Lys-Ala-Ile-Tyr-Ser, which is an integral part of the PLA2 components of the two neurotoxic complexes from *V. ammodytes meridionalis* and *V. russelli formosensis* (sequence 70–74) activated vipoxin increasing its PLA2 activity by 23%. This is in contrast to the inhibitory effect of the respective pentapeptides with 70–74 sequences on other group II PLA2s. Surprisingly, the same peptide inhibited 46% of the *V. russelli formosensis* PLA2 activity. The limited changes in the structure of the two highly homologous neurotoxins lead to considerable differences in their interaction with native peptides.

Introduction

Vipoxin is a heterodimeric neurotoxic complex of a basic strongly toxic phospholipase A2 (PLA2) and an acidic non-toxic and catalytically inactive protein component (Inh) (Tchorbanov *et al.*, 1978). It has been isolated from the venom of *Vipera ammodytes meridionalis*, one of the three subspecies of the genus *Vipera* inhabiting the Balkan peninsula (southeast Europe). The venom of *V. ammodytes ammodytes*, which is evolutionary older, contains monomeric PLA2s. Most probably, the heterodimeric vipoxin is a product of evolution of the single chain phospholipase A2 produced by the closely related *Vipera ammodytes ammodytes*. The two subunits of vipoxin, PLA2 and Inh, are highly homologous proteins, with 62% sequence identity and the same length of the polypeptide chain (Mancheva *et al.*, 1987). This is of genetic interest because vipoxin is the first reported example of a high structural similarity between an enzyme and its natural protein inhibitor. Inh reduces 60% of the PLA2 activity and considerably (3–5 times) decreases the toxicity of the basic subunit. Vipoxin is an unique example of regulation of a toxic function generated by molecular evolution which is of great pharmacological interest. The complex formation between the two subunits is absolutely necessary for the physiological function of vipoxin. When separated from the Inh, the lyo-

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philized PLA2 subunit loses irreversibly its toxicity in 3–4 days and its enzymatic activity in two weeks. On the contrary, the lyophilized complex preserves both, the toxicity and catalytic activity for several years (Aleksiev *et al.*, 1976). Evidently, the Inh plays an important stabilizing role and ensures the preservation of the biological action of the unstable PLA2 for a long period.

The crystal structure of the neurotoxin from the venom of *V. ammodytes meridionalis* at 2.0 Å (Perbandt *et al.*, 1997) and 1.4 Å (Banumathi *et al.*, 2001) resolution was previously described. The structure shows that the vipoxin complex formation is different to that of many known structures of snake venom neurotoxins. It also reveals the importance and capacity of the inhibitor for the stabilization of the biologically active PLA2.

In the present study we compare the enzymatic activity of vipoxin to that of the closely related neurotoxic complex RV4/RV7 from the venom of *Vipera russelli formosensis* as well as to the activities of the PLA2s from the venom of *Daboia russelli* and the bee venom PLA2. The inhibitory effect of aristolochic acid, vitamin E and oleyloxyethylphosphocholine toward vipoxin was also investigated. We observed an intriguing opposite effect of the peptide Lys-Ala-Ile-Tyr-Ser on the enzymatic activity of two closely related, highly homologous neurotoxic complexes from snakes inhabiting widely separated regions of the world. The peptide mentioned above is an integral part of the PLA2 components of both neurotoxins.

Materials and Methods

Neurotoxin sources

Crude venom was collected from the toxic glands of the snake *Vipera ammodytes meridionalis* inhabiting Balkan peninsula (southeast Europe). The venom was fractionated and the neurotoxic complex vipoxin isolated as described previously (Tchorbanov and Aleksiev, 1981). The toxic PLA2 subunit of vipoxin was isolated after the dissociation of the complex and purified according to the procedure given in (Mancheva *et al.*, 1986).

The neurotoxic complex RV4/RV7 from the venom of *Vipera russelli formosensis* (Taiwan Russell's viper) and the *Daboia russelli* PLA2 were obtained from the Irula Snake Farm Tamil in Nadu, India. The isolation and purification of the toxins were performed as described by Chandra *et al.*, 1999.

Chemicals

Aristolochic acid, vitamin E and oleyloxyethylphosphocholine were purchased from Calbiochem. The pentapeptide Lys-Ala-Ile-Tyr-Ser was synthesized by solid phase method in PS3 Solid Phase Peptide Synthesizer. The coupling proceeds from C-terminus, which is linked to the resin, towards the N-terminus of the peptide.

Enzyme activity

Phospholipase A2 activity was determined using the Cayman Chemical secretory PLA2 Assay kit (Ann Arbor, USA) containig a bee venom PLA2 as a standard. The substrate was 1,2 – dithio analog of diheptanoyl phosphatidylcholine. The release of free thiols upon the PLA2 catalyzed hydrolysis of the thio ester bond at the *sn*-2 position was detected spectrophotometrically using 5,5′-dithiobis (2-nitrobenzoic acid).

Results and Discussion

Enzymatic activities of two closely related neurotoxic complexes from viperidae snake venoms, vipoxin from *Vipera ammodytes meridionalis* and RV4/RV7 from *Vipera russelli formosensis*, as well as those of the PLA2 from the venom of *Daboia russelli pulchella* and the bee venom PLA2, used as a standard in the secretory PLA2 assay kit of the company Cayman Chemical (Ann Arbor, USA), were determined and compared (Fig. 1). Equimolar concentrations of the respective enzymes were used. The activity of vipoxin is 1.7, 6.6 and 4 times higher than those of the other neurotoxins. The neurotoxic complex RV4/RV7 is an analog of vipoxin. It has been isolated from the Taiwan Russell's viper venom. The sequences of the two components of the last complex, RV4 (basic and neurotoxic subunit) and RV7 (acidic, nontoxic and almost devoided of enzymatic activity polypeptide of the same length as RV4), are 92% identical to the vipoxin PLA2 and Inh, respectively (Fig. 2). It is impressive that the two highly homologous heterodimeric neurotoxins were found in the venoms of vipers inhabiting widely

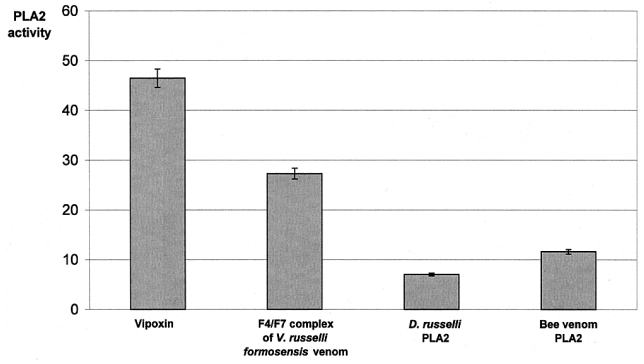

Fig. 1. Phospholipase A2 activities of neurotoxins vipoxin, RV4/RV7 and *D. russelli pulchella* PLA2 from the venoms of *Vipera ammodytes meridionalis, Vipera russelli formosensis* and *Daboia russelli*, respectively. The activity of the bee venom PLA2, a standard in the secretory PLA2 assay kit of the company Cayman Chemical (Ann Arbor, USA), is used as a reference. The concentrations of the respective PLA2s in each experiment were equimolar. The enzyme activity is presented in arbitrary units. The activity of vipoxin is 1.7, 6.6 and 4 times higher than those of the F4/F7 complex from the venom of *Vipera russelli formosensis*, *Daboia russelli* PLA2 and bee venom PLA2, respectively.

separated regions of the world: the Balkan peninsula (southeast Europe) (Tchorbanov *et al.*, 1978) and Taiwan (Asia) (Wang *et al.*, 1992). The enhanced enzymatic activity of vipoxin can be attributed to the limited structural changes in the two subunits in comparison to those of the complex RV4/RV7. The sequences of RV4 and the vipoxin PLA2 differ only in 10 amino acid residues (Fig. 2), five of the substitutions, R7K, K56R, R79K, R86K and K125R being conservative. The most radical substitutions, connected with a change of the electrostatic charge, are G54R, Q78K, T94D, E116 N and R129T. Further, 9 substitutions in the polypeptide chain of the vipoxin inhibitor were observed in comparison to that of RV7 (Fig. 2). Three of them are connected with a change of the charge: E11Q, T54R and Q108E. On the whole, vipoxin possesses two positively charged residues more than RV4/RV7 complex (Fig. 2). In particular, the substitutions G54R and Q78K in the PLA2 subunits of the heterodimers and T54R in the non-enzymatic components might be responsible for the observed changes. Arg 54 is located at the end of one of the two antiparallel helices (37–54) that forms the backwall of the substrate binding pocket. Lys 78 is a part of the crucial β-wing substructure (residues 74–84) which may adopt a variety of orientations and play an important pharmacological role (Scott, 1997). This region has also been proposed to be related to the

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                1                                                          56
Vipo-Pla2       NLFQFAKMINGKLG-AFSVWNYISYGCYCGWGGQGTPKDATDRCCFVHDCCYG RVR
RV-4            NLFQFARMINGKLG-AFSVWNYISY GCYCGWGGQGTPKDATDRCCFVHDCCYGGVK

Vipo-Inh        NLFQFGDMILQKTG-KEAVHSYAIYGCYCGWGGQGRAQDATDRCCFAQDCCYGRVN
RV-7            NLFQFGE MILEKTG-KEVVHSYAIYGCYCGWGGQGRAQDATDRCCFVHDCCYGTVN

            57                          78                             113
Vipo-Pla2   -GC-N------PKLAIYSYSFKKGNIVCGK-NNGCLRDICECDRVAANCFHQNKNTY
RV-4        -GC-N------PKLAIYSYSFQRGNIVCGR- NNGCLRT ICECDRVAANCFHQNKNTY

Vipo-Inh    -DC-N------PKTATYTYSFENGDIVCGD-NDL CLRAVCECDRAAAICLG ENVNTY
RV-7        -DC-N------PKTATYSYSFENGDIVCGD-NDL CLRTVCECDRAAA ICLGQNVNTY

        114                     133
Vipo-Pla2   NKNYKFLSS-SR CRQTSEQC
RV-4        NKE YKFLSS-SKCRQRSEQC

Vipo-Inh    DKNYEYYSI-SHCTEESEQC
RV-7        DKNYEYYSI-SHCTEESEQC
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Fig. 2. Comparison of the amino acid sequences of vipoxin from the venom of *Vipera ammodytes meridionalis* and the neurotoxic complex RV4/RV7 from the venom of *Vipera russelli formosensis* (Taiwan Russell's viper). Vipo-PLA2 and RV4 are the enzymatically active PLA2 subunits of the complexes vipoxin and RV4/RV7. Inh and RV7 are the non-enzymatic components of the complexes. Substitutions are marked.

neurotoxicity of the enzyme (Yang, 1997). Detailed inspection of the vipoxin X-ray crystal structure shows that both residues protrude out at the surface region and are not forming any intermolecular contacts. It appears that these positively charged residues, free of intermolecular interactions and protruding out at the surface of the molecule, are responsible for the enhanced activity of vipoxin. As it is already known (Kini, 1997) such residues facilitate the binding of PLA2 to aggregated lipid substrates by increasing the enzyme penetratebility into the membranes. This is important for the pharmacological potency. Phylogenetic analysis has also demonstrated that snake-venom group II PLA2 genes evolved by accelerated evolution, leading to diverse activities (Ogawa *et al.*, 1995). Two other radical substitutions in the vipoxin PLA2 of a charged residue by a neutral one and vice versa, T94D and E116N, are mutually neutralized. The same is valid for substitutions E11Q and Q108E in Inh. The substitution of the active site His 48 with Gln 48 is the reason for the absence of catalytic activity of the inhibitory component of the Vipoxin complex. All this substitutions in Vipoxin presumably improve the binding of the complex to the toxin receptors and increase the affinity and catalytic efficiency of the *V. ammodytes meridionalis* neurotoxin.

We have also investigated the effect of aristolochic acid, vitamin E and oleyloxyethylphosphocholine on the enzymatic activity of the complex Vipoxin and the separated catalytically active subunit (Table I). Inhibitors of PLA2 are expected to have a therapeutical potential and are useful in determining the biological role of these lypolitic enzymes (Gelb *et al.*, 1994). Aristolochic acid is an alkaloid from the plant *Aristolochia radix.* Plants and their extracts are used as remedies for snake venom poisoning and for neutralization of some undesired effects of PLA2s (Vishwanath and Gowda, 1987). Aristolochic acid inactivates the *Naja naja atra* and *Bungarus multicinctus* venoms (Tsai *et al.*, 1980). However, this alkaloid had no effect on the toxicity of PLA2s from *V. russelli*, *Trimeresurus mucrosquamatus*, *Agkistrodon acutus* and *T. gramineus* venoms (Vishwanath and Gowda, 1987). Aristolochic acid inhibits partially (appr. 1/3) of the phospholipase A2 activity of both vipoxin complex and the separated PLA2 subunit (Table I).

Vitamin E (D-α-tocopherol) is a physiological membrane lipid antioxidant. It has been shown that this compound inhibits platelet phospholipase A2 which suggests a regulation function of tocopherol in the arachidonate release from the membrane phospholipids and its subsequent metabolism (Douglas *et al.*, 1986). PLA2 hydrolyzes plasma membrane phospholipids releasing arachidonic acid which is a precursor of eicosanoid mediators of inflammation: leukotriens, prostaglandins and tromboxanes. In this way PLA2 is involved in inflammatory processes and diseases such as rheumatoid arthritis and asthma (Scott *et al.*, 1990). In a 50-molar excess vitamin E inhibits 35% of the vipoxin activity and 25% of the enzymatic activity of the separated PLA2 subunit (Table I). The complex formation between the neurotoxic PLA2 and non-toxic Inh facilitates the interaction with D-α-tocopherol in comparison to the separated PLA2.

In a 50-fold molar excess oleyloxyethylphosphocholine inhibits 38% of the vipoxin enzymatic activity and 15% of the separated PLA2 component activity (Table I). Again, the degree of inhibition of the complex is higher than that of the isolated subunit.

The results reported here clearly indicate that oleyloxyethylphosphocholine, like aristolochic acid and vitamin E, is capable to inhibit the enzymatic activity of the neurotoxic complex vipoxin and its PLA2 component. In this way the three compounds can decrease the rate of liberation of free arachidonic acid in biological systems and influence inflammatory processes because eicosanoid biosynthesis depends on the release of the acid mentioned above. The phospholipase A2 activity is partially suppressed which is due probably

Neurotoxin	Aristolochic acid (% inhibition)	Vitamin E (% inhibition)	Oleyloxyethylphosphocholine (% inhibition)
Vipoxin	29	35	38
Vipoxin PLA2	35	25	15

Table I. Inhibitory effect of aristolochic acid, vitamin E and oleyloxyethylphosphocholine on the phospholipase A2 activity of the neurotoxic complex vipoxin and its separated PLA2 subunit. A 50-fold molar excess of the inhibitor was used.

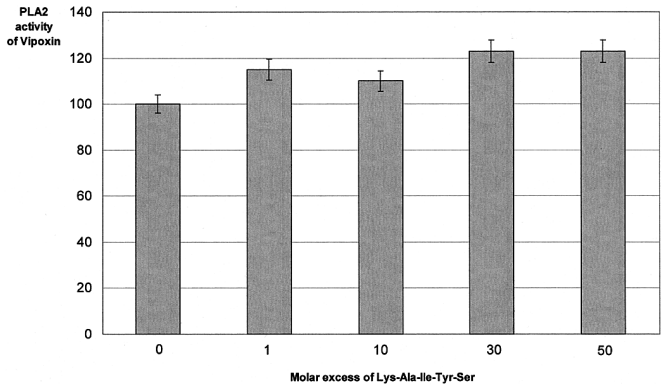

Fig. 3. Effect of the pentapeptide Lys-Ala-Ile-Tyr-Ser with the 70–74 sequence of the vipoxin PLA2 on the enzymatic activity of the neurotoxic complex.

to steric hindrances or moderate affinity of the enzyme for the inhibitors.

It has been shown that pentapeptides, which are an integral part of the native enzyme polypeptide chain, can inhibit type II PLA2s (Tseng *et al.*, 1996). Thus, peptides with the 70–74 sequence of the human type II PLA2 and related enzymes from *Crotalus durissus* and *Crotalus atrox*, which have different sequences in the 70–74 region, inhibit phospholipid hydrolysis catalyzed by these hydrolases. The inhibition by each peptide is specific to the enzyme from which the peptide sequence is derived. It has been proposed that the inhibition proceeds via a sequence-specific, noncovalent interaction with the N-terminal segment of the PLA2 polypeptide chain which forms the enzyme substrate-binding site. The 70–74 sequence is a part of a sole β-wing structure, conserved among class II secretory PLA2s and contains a tyrosyl residue which participates in the catalytic network and supports the active site structure. This curious substructure is probably connected with some pharmacological properties of PLA2 such as anticoagulation (Scott, 1997). In this study we determined the effect of pentapeptide Lys-Ala-Ile-Tyr-Ser with the 70–74 sequence of the vipoxin PLA2 on the enzymatic activity of the neurotoxic complex. This peptide activated vipoxin increasing its phospholipase A2 activity by 23% when the enzyme: peptide ratio was 1:50 (Fig. 3). This is in contrast to the inhibitory effect of the respective pentapeptides with 70–74 sequences of group II PLA2s (Tseng *et al.*, 1996). Surprisingly, we observed a 46% inhibition of the *V. russelli formosensis* PLA2 activity by the same peptide Lys-Ala-Ile-Tyr-Ser which is also an integral part (sequence 70–74) of the RV4 polypeptide chain. The limited changes in the structure of the two neurotoxic complexes (92% sequence identity) lead to considerable differences in their interaction with native peptides.

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