Localization of Structural Elements of Bee Venom Phospholipase A₂ Involved in N-type Receptor Binding and Neurotoxicity*

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We have shown previously that neurotoxic venom secretory phospholipases A₂ (sPLA₂)s have specific receptors in brain membranes called N-type receptors that are likely to play a role in the molecular events leading to neurotoxicity of these proteins. The sPLA₂ found in honey bee venom is neurotoxic and binds to this receptor with high affinity. In this paper, we have used a number of mutants of bee venom sPLA₂ produced in Escherichia coli to determine the structural elements of this protein that are involved in its binding to N-type receptors. Mutations in the interfacial binding surface, in the Ca²⁺-binding loop and in the hydrophobic channel lead to a dramatic decrease in binding to N-type receptors, whereas mutations of surface residues localized in other parts of the sPLA₂ structure do not significantly modify the binding properties. Neurotoxicity experiments show that mutants with low affinity for N-type receptors are devoid of neurotoxic properties, even though some of them retain high enzymatic activity. These results provide further evidence for the involvement of N-type receptors in neurotoxic processes associated with venom sPLA₂s and identify the surface region surrounding the hydrophobic channel of bee venom sPLA₂ as the N-type receptor recognition domain.

Secretory phospholipases A₂ (sPLA₂,s, 14 kDa) catalyze the hydrolysis of the sn-2 position of glycerophospholipids to yield fatty acids and lysophospholipids (1–3). They are found in mammalian tissues and in the venom of a wide range of organisms (insects, reptiles, amphibians, arachnids, coelenterates). Most sPLA₂s require millimolar concentrations of Ca²⁺ as a catalytic cofactor and show broad specificity for phospholipids with different polar head groups and fatty acyl chains (2, 3). sPLA₂s have been classified into three main groups according to their primary structure, including the location of their disulfide bridges (2, 4, 5). The mammalian pancreatic sPLA₂, the mammalian non-pancreatic inflammatory sPLA₂, and the sPLA₂ purified from honey bee venom (denoted bvPLA₂) are prototypes of group I, II, and III sPLA₂s, respectively. The structures of type I and II enzymes are very similar, but the structure of bvPLA₂ is similar to those of the other sPLA₂ only in its active site (6).

sPLA₂s are implicated in a diversity of biological functions. Mammalian group II sPLA₂ is involved in inflammatory processes (7–11), while pancreatic group I sPLA₂ is involved not only in digestion but is also endowed with other cellular functions such as the induction of cell proliferation (12) and smooth muscle contraction (13, 14). On the other hand, most sPLA₂s from venom are toxic enzymes: they can act as neurotoxins, myotoxins, anticoagulants, and they can also cause inflammation (15–18).

The existence of specific receptors for sPLA₂ has been demonstrated recently (19, 20). Using O₂₁ and O₂₂, two sPLA₂ purified from Taipan snake Oxyuranus scutellatus scutellatus venom, two types of receptors have been characterized. The M-type receptor is a 180-kDa protein that was first characterized in rabbit skeletal muscle (20, 21). This receptor binds various venom sPLA₂s, including O₂₁ and O₂₂, but does not bind bvPLA₂. It also binds pancreatic type and inflammatory type sPLA₂s with high affinities (1—10 nM), suggesting that these sPLA₂s are probably the endogenous ligands of this sPLA₂ receptor (21). The M-type receptor has been cloned recently from various animal species (21–24), and its molecular properties have been analyzed in detail (23, 25–29). Notably, the interaction between the M-type receptor and sPLA₂ has been studied both from the receptor side (25, 29) and from the sPLA₂ side to show that the Ca²⁺-binding loop of the sPLA₂ ligand plays a central role in binding to M-type receptor (28). Although the biological role of the M-type receptors remains to be clearly elucidated, this receptor has been proposed to be involved in many biological effects of the pancreatic sPLA₂ (30).

Another type of PLA₂ receptor has been identified in rat brain using O₂₂ as a ligand and called the N (for neuronal)-type sPLA₂ receptor (19). N-type receptors are highly expressed in brain, but are also present in heart, skeletal muscle, kidney, lung, liver, pancreas, and smooth muscle (31). They have been shown to consist of proteins of 36–51 and 85 kDa (19). N-type receptors have a pharmacological binding profile distinct from that of M-type receptors. They bind several neurotoxic sPLA₂s, including O₂₂ and bvPLA₂, with high affinity, but unlike M-

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type receptors they do not bind pancreatic and inflammatory type sPLA₂s (19, 32). These N-type receptors are thought to mediate some of the physiological, pathophysiological, and toxic effects of sPLA₂s. The normal biological role(s) of N-type receptors is presently unknown. Based on the fact that these N-type receptors bind neurotoxic sPLA₂s but not nonneurotoxic receptors is presently unknown. Based on the fact that these N-type receptors are involved in the neurotoxicity of venom sPLA₂ after intracerebroventricular injection (19, 33).

The purpose of this study is to identify the region of sPLA₂ that is responsible for both their interaction with N-type receptors and their neurotoxicity. These studies were carried out using a series of mutants of the neurotoxic bvPLA₂, a very specific ligand of the N-type receptors (32–34) and one in which its tridimensional structure has been obtained at high resolution (35).

**EXPERIMENTAL PROCEDURES**

*Materials—* O₂ was purified and iodinated as described previously (19). Pa₂ and Pa₅, two sPLA₂s from *Heloderma suspectum* were a generous gift from Prof. André Vandermeers (Université Libre de Bruxelles, Brussels, Belgium). The bvPLA₂ expression plasmid p6xHis-Kall-BV-PLA₂–#2 has been described previously (36). Technical grade guanidine hydrochloride and USP grade urea were purchased from AMRESCO (Solon, OH). The following commercial kits, GeneClean kit (BIO101, Inc., Foster City, CA), QIAgen plasmid mini-kit (Qiagen Inc., Chatsworth, CA), and DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) were used according to the manufacturer’s instructions. 

**Construction of the Recombinant bvPLA₂ Mutants—** All mutants were made using a PCR-based method. All primers used for mutagenesis are listed in Table I. Some of the primers contain a mixture of A, G, T, C, R = (A, G), S = (C, G), Y = (C, T), W = (A, T).

### Table I

| Mutant | PCR primer |
|--------|------------|
| I1C    | 5'-ATC ACA GAT CTC CGT TGC GCT GCA TCT AC |
| I2C    | 5'-ATC ACA GAT CTC ACT TCC GCA TCT GTC AC |
| I2C/H34R/V40F<sup>a</sup> | Same I2C |
| G12X (X = A, C)<sup>b</sup> | 5'-TCT ACC CGG GGA CTC TGT GGT GTG GTC ACK SCA ACA |
| G12S/K14R<sup>a</sup> | Same as G12X |
| N13C   | 5'-AAC CGG CGG AGT TCG TTC TGC GCA CAC GAT CGG |
| K14E   | 5'-AAC CGG CGG AGT TCG TTC TGC GCA CAC GAT CGG |
| S15C   | 5'-AAC CGG CGG AGT TCG TTC TGC GCA CAC GAT CGG |
| S16C   | 5'-AAC CGG CGG AGT TCG TTC TGC GCA CAC GAT CGG |
| R23C   | 5'-AAC CGG CGG AGT TCG TTC TGC GCA CAC GAT CGG |
| R23E   | 5'-GAG GAC ATG CGG TTC GTG TTA AGC RGC CG |
| T51C   | 5'-GAG ACG GTG TTA AGC AGA GGC GTT GTA TAA CCC |
| T53C   | 5'-GAG ACG GTG GAG AGA GAC GCA GTT AGA CCC |
| T57X (X = D, E, F, L, V) | 5'-CAC GGG TTA ACA AAG GCT TCT CAC KWM CGT |
| R58A   | 5'-CGG CGC TGA GGC GGT GCT ACT GC |
| K66C   | 5'-CAC ACG CTG AGC TTC GAC GTC GAC TGC TAC TAC |
| 178X (X = C, Y) | 5'-CTG CCT TAA GAA CTC CCG CCA TAC GTC TTC TTT |
| F82C   | 5'-GAT ACG AAT TCT TCT TAC TGC GTT TTG AGC RGC CG |
| K55C   | 5'-TAC GAT TCT TTA GCT TCC TGC GTG TTC CAT GTA T |
| K94E   | 5'-GTT ACC GGG TGC TCG AGT TTG TAA CAT TGC GTA T |
| I91F   | 5'-GCC GTG CAC GTC GGC AGT CGG TTT TTA GTA ACA TTT GAT CAG |
| D92C   | 5'-GCC GTG CAC GTC GGC AGT CGG TTT TTA GTA ACA TTT GAT CAG |
| K133E  | CTA ATT AAG CTT CAG TAT TGC CGC AG |

<sup>a</sup> The mutations H34R and V40F of 12C/H34R/V40F and K14R of G12S/K14R were introduced unintentionally during PCR.

<sup>b</sup> The symbols used for mixed primers are as follows: K = (G, T), M = (A, C), R = (A, G), S = (C, G), Y = (C, T), W = (A, T).

The purpose of this study is to identify the region of sPLA₂ that is responsible for both their interaction with N-type receptors and their neurotoxicity. These studies were carried out using a series of mutants of the neurotoxic bvPLA₂, a very specific ligand of the N-type receptors (32–34) and one in which its tridimensional structure has been obtained at high resolution (35).
changed to cysteine require special comment. Analysis of these mutants with Ellman's reagent after refolding them in the presence of the cysteine/cystine redox couple buffer showed that in all cases these proteins lack free SH groups. This suggests that not only have all the 10 cysteines of wild-type bvPLA₂ formed disulfides, but the added cysteines are engaged in a disulfide bond with free cysteine from the buffer. This result was confirmed by analysis of the mutants by mass spectrometry and by the fact that treatment of these mutants with DTT under conditions that do not cleave the disulfide of wild-type enzyme results in the appearance of 0.7–1.0 equivalents of SH/mole of enzyme.

The full details of these analyses will be reported elsewhere.

Preparation of Deglycosylated bvPLA₂—Natural purified bvPLA₂ (300 µg) was incubated with or without 1 unit N-glycosidase F (Boehringer Mannheim, catalog number 913 782) in 100 mM sodium phosphate buffer (pH 7.4) containing 10 mM EDTA for 18 h at 37°C. After digestion, the mixtures were chromatographed on a C18 Waters column (4.6 x 250 mm) prewarmed at 30°C with a Beckman system gold apparatus. Elution was performed using an acetonitrile linear gradient in 0.5% trifluoroacetic acid, 0.9% triethylamine, 0.001% t-butyl mercaptan, 27–40% acetonitrile for 35 min at 1.4 mL/min. The eluted fractions were lyophilized, resuspended in water, and assayed for protein concentration by measurement of the absorbance at 280 nm. 1.5 µg of the samples were then analyzed by high resolution SDS/Tris/Tricine gels (16.5%) under reducing conditions followed by Coomassie Blue staining. Protein molecular mass markers were from Promega.

Binding Experiments—All N-type receptor binding experiments were performed as described (19). Briefly, membranes, 125I-OS₂, and competitors were incubated at 20°C in 0.5 or 1 mL of buffer (140 mM NaCl, 0.1 mM CaCl₂, 20 mM Tris, pH 7.4, and 0.1% bovine serum albumin). Incubations were started by addition of rat brain membranes and filtered after 90 min of incubation through GF/C glass fiber filters presoaked in 0.5% polyethyleneimine.

Toxicity Experiments—Neurotoxic properties of the various recombinant sPLA₂s have been determined by intracerebroventricular injections in adult male Balb/C mice (average weight, 20 g). Animals were anesthetized using a mixture of ketamine hydrochloride (100 mg/kg, Rhône Merieux) and xylazine hydrochloride (12 mg/kg, Bayer Pharma). Injections of sPLA₂s in a volume of 5 kDa was observed between the two main peaks that is consistent with the molecular mass of the carbohydrate moiety of bvPLA₂ made of 14 N-glycans (42). This suggests the major peak (peak B) effectively corresponds to deglycosylated bvPLA₂. Furthermore, the migration position of the faster moving protein is identical to that of the non-glycosylated fraction that is present in natural bee venom (36). The identification of peak B as deglycosylated bvPLA₂ was further confirmed by N-terminal protein sequencing, indicating that Asn-13 has been fully deglycosylated (not shown). Results from competition binding experiments with deglycosylated bvPLA₂ for binding to N-type receptors are shown in Fig. 2C. Interestingly, deglycosylated bvPLA₂ displays a Kₐ₅₀ value nearly identical to that of the cleaved recombinant bvPLA₂ (Fig. 1). This Kₐ₅₀ value is about five times higher than that of the natural glycosylated bvPLA₂. Taken together, these results indicate that the carbohydrate moiety of bvPLA₂, although not crucially involved in the interaction of bvPLA₂ with N-type receptors, increases the affinity by a factor of about 5.

Analysis of Mutations in the Putative Interfacial Recognition Surfaces of bvPLA₂—The x-ray crystal structures of group I–III sPLA₂s containing a bound phospholipid analog reveal a common constellation of catalytic residues lying about two-thirds down the length of the active site slot where the substrate binds (6). Despite these common features, the global topology of bvPLA₂ is very different than those of group I and II sPLA₂s. It is currently thought that the surface of bvPLA₂ that contacts the membrane bilayer, the interfacial recognition surface (IRS), surrounds the opening of the active site slot (Figs. 3 and 4). This putative IRS of bvPLA₂ is composed of residues that lie on one face of an α-helix (composed of residues 76–91), of the carbohydrates of bvPLA₂, and of the molecular determinants of sPLA₂ that are implicated in binding to N-type receptors. Furthermore, as both uncleaved fusion protein and cleaved recombinant bvPLA₂ bind nearly equally well to N-type receptors, the different mutants have been used as recombinant proteins containing the N-terminal peptide extension.

Fig. 1 indicates a 3–6-fold difference in affinity between recombinant and natural bvPLA₂. Natural bvPLA₂ is heavily glycosylated on Asn-13 (41, 42), but the recombinant forms of bvPLA₂ have been produced in Escherichia coli where glycosylation does not occur. This suggests that differences in affinity

![FIG. 1. Inhibition of 125I-OS₂ binding to N-type receptors by recombinant bvPLA₂s produced in E. coli. Binding experiments were performed as described under “Experimental Procedures.” Results are expressed as the percentage of control that corresponds to 125I-OS₂-specific binding measured in the absence of competitor. Kₐ₅₀ values measured for natural bvPLA₂ (BV), for the recombinant bvPLA₂ expressed as a fusion protein (rBVp), and for the cleaved recombinant bvPLA₂ (∆BV) are indicated in the figure.](image-url)
**Interaction of Neurotoxic Bee Venom PLA<sub>2</sub> with Its Receptor**

For these studies, a number of mutants were made in which the wild-type residue was replaced with cysteine. During *in vitro* refolding of mutants, cysteine and cystine are present in the buffer to provide a redox couple that favors formation of the five disulfide bridges that are present in natural bvPLA<sub>2</sub>. After refolding of all mutant proteins containing the desired additional cysteines, the cysteines end up in a disulfide linkage to a free cysteine from the redox buffer. This is useful for initial studies aimed at mapping the receptor recognition surface of bvPLA<sub>2</sub>, since the wild-type residue is replaced by a fairly large residue that is also zwitterionic at physiological pH and thus very hydrophilic.

Replacement of Ile-1 and Ile-2 by cysteine (I1CC and I2CC, respectively, where CC denotes the protein’s cysteine disulfide linked to a free cysteine) results in a 20–25-fold increase in *K<sub>0.5</sub>* value (i.e., a decrease in receptor affinity) as compared with that of wild-type bvPLA<sub>2</sub>, suggesting that these two positions play a role in recognition of N-type receptors (Table II). Mutations of surface residues located within the 76–91 helix also lead to reduced binding properties. Mutations of Lys-85 into cysteine (K85CC) or glutamic acid (K85E) reduce the affinity by a factor of about 40 (Table II). Binding properties of double and triple mutants involving K85E have also been analyzed. When Lys-85 is mutated together with either Lys-133 (K85E/K133E), Arg-23 and Lys-133 (R23E/K85E/K133E), or with Lys-94 and Lys-133 (K85E/K94E/K133E), a dramatic decrease in receptor binding was observed, especially for triple mutants (Table II). Mutations of hydrophobic surface residues of the 76–91 helix also lead to a significant decrease in the binding affinity of bvPLA<sub>2</sub> to N-type receptors. Mutation of Ile-91 into phenylalanine (I91F) leads to a mutant with a binding affinity of bvPLA<sub>2</sub> to N-type receptors. Mutation of Ile-78 and Phe-82 to cysteine (D92CC) have only a minor effect on the binding activity.

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**Table II**

| K<sub>0.5</sub> value | Binding activity, % of rvBV | aPLA<sub>2</sub> activity<sup>a</sup> | bPLA<sub>2</sub> activity<sup>a</sup> |
|-----------------------|-----------------------------|-------------------------------|-------------------------------|
| rbvPLA<sub>2</sub>,fp | 2.0 | 100 | |
| rbvPLA<sub>2</sub> | 4.6 | 170 | |
| bPLA<sub>2</sub> (purified) | 0.8 | 183 | |

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<sup>a</sup> All of the mutants were also assayed using the well-established pH-stat method with vesicles of the anionic phospholipid 1,2-dimyristoylphosphatidylcholine (36). The results are essentially the same within 20%, as those obtained with the fluorimetric assay. In both assays, all of the mutants are tightly bound to vesicles, and thus the decrease in catalytic turnover is due to an increase in the interfacial *K<sub>0.5</sub>*, a decrease in the interfacial *k<sub>cat</sub>*, or a combination of both.

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**Fig. 2. Preparation and binding properties of deglycosylated bvPLA<sub>2</sub>**. A, elution profile of natural bvPLA<sub>2</sub> treated with N-glycopeptide F on C18 reverse phase column. Retention time for bvPLA<sub>2</sub> treated without N-glycopeptide F corresponds to peak A (not shown). B, gel analysis of untreated bvPLA<sub>2</sub> N-glycopeptide F/bvPLA<sub>2</sub> reaction mixture, and peaks A and B. C, comparison of binding properties to N-type receptors for natural glycosylated bvPLA<sub>2</sub> (○) and purified deglycosylated bvPLA<sub>2</sub> (●). Binding conditions are as indicated in the legend to Fig. 1.
that increase

Despite this very different localization in the primary sequence, the conformation of the bvPLA2 Ca\(^{2+}\)-binding loop is strikingly similar to that of group I and group II sPLA2s, and it contains the consensus motif X-Cys-Gly (35).

Mutations of Asn-13 and Ser-15 to cysteine (N13CC and S15CC, respectively) have a weak effect on the receptor binding properties of bvPLA2 (Fig. 5B and Table II). Mutation of Lys-14 to glutamic acid (K14E) or to cysteine (K14CC) also have a modest effect on receptor binding, although the double mutant K14E/R23E binds 580-fold less tightly to N-type receptor. Mutations at position 12 have interesting consequences. Replacement of Gly-12 with the hydrophilic residues cysteine (G12CC) or serine (together with a conservative mutation of Lys-14 into arginine, which was inadvertently introduced during PCR) (G12S/K14R) weakens receptor binding only slightly. However, mutation of Gly-12 to the hydrophobic residue alanine (G12A) results in a protein that does not detectably bind to N-type receptors, suggesting that the N-type receptor donates a role in receptor binding. Mutation of lysines 66 and 72, which are located on the side of bvPLA2 opposite of the IRS, to either cysteine (K66CC and K72CC) or glutamic acid (K66E) has no effect on the K\(_{0.5}\) of receptor binding (Table II), suggesting that this region of the molecule is not involved in binding to N-type receptors. Similarly, mutation of Lys-122 and Lys-133 to glutamine (K122Q) or glutamic acid (K133E) produces only modest effects on receptor binding (Table II). Conversely, mutation of Lys-94 to glutamic acid leads to a moderate but significant decrease in the binding affinity of the mutant bvPLA2 relative to the wild-type enzyme (Table II). This residue corresponds to the active site residue Asp-99 of group I and group II sPLA2s, which is conserved in all catalytically active enzymes including both neurotoxic and nontoxic sPLA2s (5). Mutation of Asp-64 to alanine (D64A) results in a dramatic decrease in receptor affinity (K\(_{0.5}\) = 1 \(\mu\)M). However, more conservative substitutions of Asp-64 by glutamic acid (D64E) or by asparagine (D64N) do not lead to a dramatic decrease in receptor affinity (Fig. 5C). Finally, a double mutant where Asp-64 is replaced by a glutamic acid (D64E) together with a mutation at position 66 (D64E/K66CC, D64E was introduced inadvertently during the preparation of K66CC by PCR) also retains a high affinity for N-type receptors (Table II).

Analysis of Deletions and Other Mutations—bvPLA2 is a basic enzyme (pI = 8.04) that has a number of lysine and arginine residues on its surface that may interact with N-type receptors. This protein also contains several solvent-exposed threonine and serine residues that might also have a role in receptor binding. Mutation of lysines 66 and 72, which are located on the side of bvPLA2 opposite of the IRS, to either cysteine (K66CC and K72CC) or glutamic acid (K66E) has no effect on the K\(_{0.5}\) (Table II), suggesting that this region of the molecule is not involved in binding to N-type receptors. Similarly, mutation of Lys-122 and Lys-133 to glutamine (K122Q) or glutamic acid (K133E) produces only modest effects on receptor binding (Table II). Conversely, mutation of Lys-94 to glutamic acid leads to a moderate but significant decrease in binding affinity of the mutant bvPLA2 relative to the wild-type enzyme (Table II). Similarly, mutation of Arg-58 into alanine (R58A) also results in a decrease of the K\(_{0.5}\) value, the corresponding K\(_{0.5}\) value is more than 35 times higher than that of the wild-type enzyme. In addition, a synergistic effect was observed with the double mutant R23E/R58A in that this protein did not detectably bind to N-type receptors (Table II). This result is not surprising since Lys-94 and Arg-58 are located in the vicinity of the 76–91 helix, and residues of this helix have already been shown to be involved in receptor binding (see above). Threonines 51 and 53 are located on the surface of bvPLA2, but far away from the IRS (Fig. 3). As expected, mutation of these residues into cysteine (T51CC and T53CC) fail to produce drastic effects on receptor binding (Table II).
Basic residues are also found on the surface of the bvPLA₂, in a loop located between the Ca²⁺ binding site and the α-helix containing the active site residue His-34 (Fig. 3). Relative to the IRS, this loop lies toward the center of the globular protein, at the left edge of the structure, and thus the amino acid side chains of this loop are relatively far from the Ca²⁺ loop and from the IRS. When Lys-25 of this loop was mutated to glutamine, the corresponding mutant (K25Q) retains binding properties (K₀.₅ = 2.7 nM) very similar to that of wild-type recombinant bvPLA₂, indicating that this position is not involved in binding to N-type receptors (Fig. 5D). Mutations of the neighboring Arg-23 and Phe-24, which are located closer to the IRS than Lys-25 (Fig. 3), into either glutamic acid (R23E and F24E, respectively) or cysteine (R23CC and F24CC) result in a significant decrease in receptor binding (Fig. 5D and Table II), consistent with the results obtained with the double mutant K14E/R23E (see above).

We have also analyzed the role of the β-sheet-like structure composed of residues 99–118 of bvPLA₂ (Fig. 3) in receptor binding. Interestingly, a similar structure is also found in group I and group II sPLA₂s (2, 5), but no role has been proposed for this domain in supporting the enzymatic activity or toxicity of sPLA₂s. When this β-sheet domain is deleted (Δ99–118), the mutant bvPLA₂ binds to N-type receptors with an affinity (K₀.₅ = 7.6 nM) that is similar to that of wild-type protein. This result clearly indicates that the β-sheet-like structure does not interact with N-type receptors.

Is bvPLA₂ Enzymatic Activity Required for Binding to N-type Receptors?—At first glance, Table II shows that most of the mutants having weak receptor binding properties also have low enzymatic activity. However, a detailed comparison of the data indicates that receptor binding and enzymatic activity are two independent molecular events. Indeed, a clear dissociation between binding and catalytic activity can be found with various mutants of the Ca²⁺-binding loop and the IRS. For example, G12A has no measurable affinity for N-type receptors, whereas it retained 37% enzymatic activity as compared with wild-type enzyme. K85E/K133E binds to N-type receptors 180-fold weaker than wild-type enzyme, but this mutant retains 69% enzymatic activity. K14CC and K85CC have reduced binding properties with K₀.₅ values of 42 and 69 nM, respectively, while they still have high enzymatic activity (29 and 42% relative to wild-type, respectively). The F82CC mutant also shows a lack of correlation in receptor binding and enzymatic activity, but to a lesser extent (280 nM and 7% enzymatic activity). These results indicate that binding to N-type receptors and catalytic activity are independent events, although several residues of the Ca²⁺-binding loop and of the IRS are required for both activities.

Relationships between Neurotoxicity, Binding to N-type Receptors, and Enzymatic Activity—The neurotoxicity of venom sPLA₂s, including bvPLA₂, has been previously suggested to be associated with their binding to N-type receptors (19, 33). This is based on the observation that the neurotoxicity of a collection of sPLA₂s correlates reasonably well with their affinity for N-type receptors. The conclusions from these previous studies would be strengthened by carrying out neurotoxicity studies with point mutant bvPLA₂s that retain high enzymatic activity but fail to bind to N-type receptors and those proteins with the vice versa properties. Studies along these lines are summarized in Table III. For these studies, we have chosen bvPLA₂ mutants that show a clear dissociation between enzymatic activity and receptor affinity. The data in Table III indicate that recombinant wild-type bvPLA₂ expressed in bacteria as a fusion protein is lethal to mice at very low doses of 25–125 nmol/kg when injected by the intracerebroventricular route. The behavioral symptoms occurring after injection were periods of catalepsy while the mice showed breathing problems, and these events were separated by periods of violent convulsions lasting from 10 to 60 s. These symptoms are very similar to those observed after injection of natural bvPLA₂ into rat brain (33). These symptoms were also observed with all of the mutants showing neurotoxicity. K14CC and K85CC are mutants that retain a high degree of bvPLA₂ enzymatic activity (within 4-fold of wild-type enzyme), whereas their K₀.₅ values for binding to N-type receptors are increased by a factor of 20–30 relative to that of the wild-type protein. The data in Table III show that these mutants are still lethal, although the effective doses needed to kill mice are much higher. Indeed, 125 nmol/kg of K14CC killed only three out of six mice, while identical or lower quantities of wild-type bvPLA₂ killed all or almost all of
Similarly, 250 nmol/kg of K85CC is needed to kill four out of nine injected mice, while injections of a 5-fold lower amount of wild-type enzyme killed five out of six mice. The neurotoxic properties of mutants with very weak or no measurable affinity for N-type receptors were also analyzed. Importantly, some of these mutants still display a high enzymatic activity relative to wild-type bvPLA2 (Table III). None of these mutants were lethal in mice, even at the highest doses that are possible to inject (125 nmol/kg for K85E/K133E and 250 nmol/kg for G12A and F82CC). Finally, as a control, we checked for the lethal properties of a double mutant (D64E/K66CC) that has both high enzymatic activity and high affinity for N-type receptors (Table III). This mutant displays a toxicity very similar to that of the wild-type recombinant bvPLA2, suggesting that this region of the molecule is not required to invoke lethality of bvPLA2 in mice. Altogether, these results demonstrate that the neurotoxicity of bvPLA2 is related to its ability to bind to N-type receptors and not to its ability to hydrolyze phospholipids.

DISCUSSION

The aim of this paper was 2-fold: to determine the region(s) of bvPLA2 involved in binding to N-type receptors and to determine if binding of bvPLA2 to N-type receptors and/or the enzymatic activity of this protein are required for the neurotoxic effects of bvPLA2. As a first approach to identify the region(s) of sPLA2 involved in binding to N-type receptors, we performed sequence comparisons between sPLA2s that bind or do not bind to these receptors. Two different sets of sequence comparisons were made. In the first set, we aligned a large number of group I and group II sPLA2s, including OS1 and OS2, which have a relatively high level of sequence identity but have distinct receptor binding properties (28). A second set of sequence alignments was made with group III sPLA2s, since bvPLA2 is a typical member of this group and since it also binds with very high affinity and specificity to N-type receptors. Other members of group III sPLA2s have been purified from the venom of the Gila monster lizard H. suspectum (45, 46) and of the Mexican beaded lizard Heloderma horridum horridum (47) as well as from the mediterranean medusa Rhopilema nomadica (48). Of interest for our study are the two sPLA2s, namely Pa2 and Pa5, which are major components of Gila monster (H. suspectum) venom and which have been entirely sequenced (46). These two sPLA2s display an overall identity of 38% with the bvPLA2, the level of identity increasing to 58% when the Ca2^2+-binding loop and the active site region were compared. Competition experiments between labeled OS2 and Pa2 and Pa5 for binding to N-type receptors have indicated that these H. suspectum sPLA2s do not bind to N-type receptors (not shown), suggesting that the molecular determinants for binding to these receptors are located within residues that are distinct in the structure of sPLA2s from Heloderma and bee venoms. Despite a detailed analysis of all these sequence alignments, it was essentially impossible to predict with any accuracy the region(s) of sPLA2 that is most probably involved in the interaction with N-type receptors. This led us to the present studies using site-specific mutants of bvPLA2, because this protein is conveniently expressed in functional form in bacteria (36), its high resolution x-ray structure is known (35), and it binds with high affinity to N-type receptors (19). It may be noted that when the sequence of Pa5 is overlaid onto the...
TABLE III

| sPLA2          | Binding activity | Enzymatic activity | Amount injected | Lethality (L/d) |
|---------------|------------------|--------------------|-----------------|----------------|
| rBV           | 2.0              | 100                | 25              | 1/3            |
| K14C          | 42               | 29                 | 125             | 3/6            |
| K56C          | 69               | 42                 | 250             | 4/9            |
| K56E/K133E    | 360              | 69                 | 125             | 0/4            |
| G12A          | >1000            | 37                 | 250             | 0/5            |
| F82C          | 280              | 7                  | 250             | 0/8            |
| K66C/D64E     | 2.7              | 85                 | 35              | 3/3            |

structure of bvPLA2, it is apparent that the putative IRS of the Gila monster enzyme lacks most of the basic residues found on the IRS of bvPLA2. This may be the reason that Pa5 fails to bind to N-type receptors, since results of the present study indicate that basic residues on the IRS of bvPLA2 are important for receptor binding.

A particular feature of the bvPLA2 is the presence of a high-mannose carbohydrate motif N-linked to Asn-13 (42). A possible role of this motif in binding to N-type receptors was suggested by comparison of the binding data obtained with recombinant and natural glycosylated bvPLA2. The five times lower affinity observed with fully deglycosylated bvPLA2 indicates that the carbohydrate moiety contributes to N-type receptor interaction, but only to a minor extent. Therefore, elements of recognition of N-type receptors are clearly within the bvPLA2 protein sequence.

As shown in Figs. 3 and 4, mutations that dramatically reduce the affinity of bvPLA2 to N-type receptors are segregated on the same face of the protein, namely on the 76–91 helix (especially positions 78 and 82), the Ca2+-binding loop, and regions of the N terminus of the protein. These are the same regions that make up the putative IRS that is thought to anchor the enzyme to the membrane interface (35). It is very likely that specific residues of the IRS directly contact the N-type receptor rather than the membrane to which the receptor is embedded. This is based on the observation that several point mutations such as G12A which retain good enzymatic activity in a membrane based assay, and thus bind well to membranes, fail to bind to N-type receptors. The dramatic effect observed on binding to N-type receptors after the single mutation of Gly-12 to Ala-12 suggests that position 12 is crucially involved in the interaction with N-type receptors. Interestingly, Gly-30 of porcine pancreatic sPLA2, which is analogous to Gly-12 of bvPLA2, is involved in the binding to M-type receptors (28).

The observation that residues of the Ca2+-binding loop that point away from the protein are important for receptor binding has to be considered along with the previous observation that the binding of OS2 to N-type receptors requires micromolar amounts of Ca2+ (19). It is not yet clear whether this Ca2+-dependence is due to the binding of Ca2+ to the receptor, to sPLA2, or to both. All of the Ca2+-binding loop mutants examined in the present study retain high enzymatic activity, which implies that they all bind Ca2+. Thus, the failure of mutants such as G12A to bind to N-type receptors is not due to a lack of binding of Ca2+ to the bvPLA2 mutant.

In general, mutations in other regions of bvPLA2 besides the IRS do not drastically affect the binding to N-type receptors. The exceptions are mutations at positions 57 and 64, located in the active site slot near the center of the globular protein and amino acids substitution at these positions dramatically reduce receptor binding. One interpretation of these surprising results is that a portion of the N-type receptor penetrates the active site slot and directly contacts Thr-57 and Asp-64. However, it is also possible, and perhaps more likely, that positions 57 and 64 mutants have structural alterations that propagate out to the surface of the protein. Key observations are obtained with the mutants D64N and D64A. Asp-64 of wild-type bvPLA2 is hydrogen-bonded to the NH of His-34 that faces away from the active site slot. When this residue is replaced with the iso-structural Asn, the D64N mutant retains good receptor binding affinity. The same result is obtained with D64E/K66CC. However, the D64A mutant fails to bind to N-type receptors almost certainly, because loss of the Asp-64-His-34 hydrogen bond results in nonlocalized structural changes that affect receptor binding.

Results obtained with the deletion mutant A99–118 are interesting in light of the fact that removal of such a large structural domain of bvPLA2 has essentially no effect on the enzymatic activity or the receptor binding affinity of the mutant. Furthermore, this β-sheet structure, which lies adjacent to the IRS, is found in group I, II, and III sPLA2s, suggesting that it has been conserved since the divergence between vertebrates and insects (5). The biological role, if any, of this domain remains to be established.

Since bvPLA2 is neurotoxic, has high enzymatic activity, and also binds with high affinity to N-type receptors that have been suggested previously to be involved in neurotoxicity of venom sPLA2s (19), it was important to probe the relationships between the receptor binding properties of bvPLA2 mutants and their neurotoxicity (as measured by the evaluation of their lethality in mice). Table III indicates that the residues of bvPLA2 that are involved in its lethal properties are located on the surface of the enzyme, namely in the 76–91 helix and in the Ca2+-binding loop. Mutations that reduce binding to N-type receptors also reduce the lethal properties, even when the mutation does not significantly decrease the lipolytic enzymatic activity of the mutant. This result indicates that the neurotoxicity of bvPLA2 is closely related to its affinity for N-type receptors rather than to its enzymatic activity.

Previous structure-function studies using toxic group I and group II sPLA2s have suggested the involvement of two distinct regions of sPLA2 sequences in conferring neurotoxicity to these enzymes. Comparisons of primary structures of a large number of sPLA2s have suggested that the N-terminal region of sPLA2 is involved in neurotoxicity (49). This notion is consistent with the result of the present study. In accordance with this hypothesis, chemical modifications of residues located in the N-terminal region of notexin led to a decrease in its lethal properties (50, 51). However, other studies also based on sequence comparisons have suggested that the hydrophobic region located at the C-terminal end (residues 80–110) of neurotoxic sPLA2 is involved in neurotoxicity (52). This second hypothesis is supported by results of chemical modifications of notoxin on tryptophan residues (53) and by immunological approaches using antipeptide antibodies directed against the C-terminal part of ammodytoxin A and crotoxin (54, 55).

In conclusion, this work has shown that residues of the IRS of bvPLA2 (Ca2+-binding loop and 76–91 helix) are implicated both in binding to N-type receptors and in neurotoxicity, strongly suggesting that toxicity is related to binding to N-type receptors. Whether other venom sPLA2s belonging to group I/II also use similar residues to bind to N-type receptors and to exert neurotoxicity remains to be elucidated.

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