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Neurotoxicity and other pharmacological activities of the snake venom phospholipase A_2 OS_2: The N-terminal region is more important than enzymatic activity

Morgane Rouault[1], **Lachlan D. Rash**[1], **Pierre Escoubas**[1], **Eric Boilard**[1], **James Bollinger**[2], **Bruno Lomonte**[3], **Thomas Maurin**[4], **Carole Guillaume**[5], **Stéphane Canaan**[6], **Christiane Deregnaucourt**[5], **Joseph Schrével**[5], **Alain Doglio**[4], **José María Gutiérrez**[3], **Michel Lazdunski**[1], **Michael H. Gelb**[2], and **Gérard Lambeau**[1,Δ]

[1]Institut de Pharmacologie Moléculaire et Cellulaire, CNRS-UMR 6097, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France

[2]Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, USA

[3]Universidad de Costa Rica, Faculdad de Microbiologia, Instituto Clodomiro Picado, San José, Costa Rica

[4]Laboratoire de Virologie, IFR50, Faculté de Médecine, 06107 Nice cedex 2, France

[5]USM 0504, Biologie Fonctionnelle des Protozoaires, Laboratoire de Biologie Parasitaire, Museum National d'Histoire Naturelle, 61 rue Buffon, 75231 Paris cedex 05, France

[6]Laboratoire d'Enzymologie Interfaciale et de Physiologie de la Lipolyse, CNRS-UPR 9025, 31 Chemin Joseph-Aiguier, 13402 Marseille cedex 20, France

Abstract

Several snake venom secreted phospholipases A_2 ($sPLA_2$s) including OS_2 exert a variety of pharmacological effects ranging from central neurotoxicity to anti-HIV activity by mechanisms that are not yet fully understood. To conclusively address the role of enzymatic activity and map the key structural elements of OS_2 responsible for its pharmacological properties, we have prepared single point OS_2 mutants at the catalytic site and large chimeras between OS_2 and OS_1, an homologous but non toxic $sPLA_2$. Most importantly, we found that the enzymatic activity of the active site mutant H48Q is 500-fold lower than that of the wild-type protein, while central neurotoxicity is only 16-fold lower, providing convincing evidence that catalytic activity is at most a minor factor that determines central neurotoxicity. The chimera approach has identified the N-terminal region (residues 1–22) of OS_2, but not the central one (residues 58–89), as crucial for both enzymatic activity and pharmacological effects. The C-terminal region of OS_2 (residues 102–119) was found to be critical for enzymatic activity, but not for central neurotoxicity and anti-HIV activity, allowing us to further dissociate enzymatic activity and pharmacological effects. Finally, direct binding studies with the C-terminal chimera which poorly binds to phospholipids while it is still neurotoxic, led to the identification of a subset of brain N-type receptors which may be directly involved in central neurotoxicity.

ΔAddress correspondence to: Gérard Lambeau, Institut de Pharmacologie Moléculaire et Cellulaire, CNRS-UMR 6097, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France, Tel. +33 (0) 4 93 95 77 33; Fax. +33 (0) 4 93 95 77 08; lambeau@ipmc.cnrs.fr.

Secreted phospholipases A_2 ($sPLA_2$s) comprise a large family of structurally conserved enzymes that catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release free fatty acids and lysophospholipids. These enzymes were first discovered in insect and snake venoms, and subsequently in mammalian tissues, plants, bacteria, fungi and viruses (1–6). Most $sPLA_2$s share a common set of structural features including a relatively small molecular mass (14–19 kDa), a compact structure with several disulfides, and a conserved Ca^{2+}-dependent catalytic mechanism.

To date, up to 12 different $sPLA_2$s that belong to three main structural collections have been identified in mammals (1,[7]–9). Despite an important knowledge accumulated at the molecular level (1,[9]–11), the exact biological functions of most mammalian $sPLA_2$s remain to be elucidated. Known functions include lipid digestion, host defence and lipid mediator production during normal and inflammatory processes. Some of these biological roles have been attributed to the first identified $sPLA_2$s, namely the group IB, IIA, V and X enzymes. Almost nothing is known about the biological functions of the more recently identified $sPLA_2$s IID, IIE, IIF, III, XIIA and XIIB (8,12).

Much more is known about the pharmacological effects of snake venom $sPLA_2$s. More than 280 venom enzymes have been reported over several decades (http://sdmc.lit.org.sg/Templar/DB/snaketoxin_PLA2/index.html.), and many of them display a wide spectrum of toxic effects including neurotoxic, myotoxic, cardiotoxic, cytotoxic, anticoagulant, convulsant, hypotensive, and pro-inflammatory effects (2,[13]–16). *In vitro*, they can also modulate cell migration and cell proliferation (17,18), have antibacterial activity (19), and display potent inhibitory effects against HIV-1 (20) and *Plasmodium falciparum*, the most deadly malaria parasite (21). Because of this tremendous array of pharmacological effects, it is possible that mammalian $sPLA_2$s may exert much more diverse functions than those currently known (22).

Although many venom $sPLA_2$s share similar structural features and catalytic activity, not all venom enzymes exhibit all of the above pharmacological effects (2). Furthermore, some venom $sPLA_2$s have no catalytic activity while they exert various toxic and pharmacological effects (14,[16],[18]–20). The absence of direct correlation between catalytic activity and pharmacological effects has led to the hypothesis that the specific actions of $sPLA_2$ is due to the presence of pharmacological sites on the $sPLA_2$ surface which are separated from or overlap with the catalytic site. These pharmacological sites would allow the $sPLA_2$ to bind specifically to soluble proteins or membrane-bound proteins that participate to the sPLA2 mechanism of action (23,24). Since this hypothesis was proposed, a collection of binding proteins has been identified using several toxic snake venom $sPLA_2$s (25–28). Besides β-bungarotoxin (29), early studies with the neurotoxic $sPLA_2$ OS_2 from the Australian Taipan snake *Oxyuranus scutellatus scutellatus* have led to the identification of two families of binding proteins called N- and M-type receptors (25,30). The N-type receptors bind OS_2 with picomolar affinities, are present in mammalian brain and other tissues, and are made up of multiple proteins with molecular masses of 18–24 kDa, 36–51 kDa and 85 kDa. Other neurotoxic $sPLA_2$s bind to the N-type receptors with high affinities while non toxic $sPLA_2$s including OS_1 bind with much lower affinities, suggesting that these receptors are involved in $sPLA_2$ central neurotoxicity. Conversely, the M-type receptor, which binds with high affinity both toxic and non toxic $sPLA_2$s including OS_2 and OS_1, consists of a single protein of 180 kDa, and belongs to the C-type lectin superfamily (25). Importantly, the M-type receptor binds several mammalian $sPLA_2$s (31,32), suggesting that these proteins are the endogenous ligands for this receptor, and possibly for the collection of binding proteins initially identified with venom $sPLA_2$s. Binding studies with ammodytoxins from the long-nosed viper *Vipera ammodytes ammodytes* have also led to the identification and characterization of several proteins in the brain that appear related to N- and M-type receptors ((27) and references therein).

Despite the identification of the above binding targets and many structure-function studies based on amino acid modification, site-directed mutagenesis and structural comparison (33–36), the molecular mechanisms underlying many of the pharmacological actions of venom $sPLA_2$s are not yet fully understood (2,[14],[37],38). In particular, the neurotoxicity of venom $sPLA_2$s, which has been one of the most studied pharmacological effects, is currently thought to involve both catalytic activity and binding to target proteins on presynaptic membranes (39,40). The assumption that enzymatic activity is required for neurotoxicity is however essentially based on old studies showing that alkylation of the active site histidine of several neurotoxic $sPLA_2$s by p-bromo-phenacyl-bromide substantially decreases their neurotoxicity (41–43). However, there was a greater loss of enzymatic activity than neurotoxicity, suggesting that other factors must also contribute to neurotoxicity. The fact that alkylation of different $sPLA_2$s by p-bromo-phenacyl-bromide leads to conformational changes at the surface of the enzyme (44–46) raises the possibility that the loss of neurotoxicity may not be due to a decrease of catalytic activity *per se*, but rather to an altered ability of the enzyme to interact with high affinity with its specific binding protein target on neuronal membranes. On the other hand, significant efforts have been made to identify the $sPLA_2$ pharmacological site involved in neurotoxicity, but its exact location still remains ill-defined (2,[33],[35],47). In the case of ammodytoxins, a series of structure-function studies have shown that residues located at both the N-terminal and C-terminal regions of these $sPLA_2$s are important for central neurotoxicity ((47) and references therein).

As indicated above, OS_2 has initially served as a key tool to identify the N- and M-type $sPLA_2$ receptors (25,30). It is highly neurotoxic in mice and rats (30,48), blocks acetylcholine release in *Aplysia* neurons (49), triggers cell migration (17), potentiates proinflammatory cellular signaling (50), and we report here that it can also affect chick neuromuscular transmission, be myotoxic, and exert anti-HIV and antimalarial activities *in vitro*. The main purpose of this work was thus to determine more conclusively the role of catalytic activity, to identify the location of the OS_2 pharmacological sites responsible for central neurotoxicity and other pharmacological effects, and to analyze the relationship with M-type and N-type receptors. Based on the accumulated knowledge on $sPLA_2$ catalytic activity and the complexicity of the reaction (51), we reasoned that the best way to probe the role of enzymatic activity was to produce OS_2 derivatives with single point mutations in the active site, rather than on the interfacial binding surface of the $sPLA_2$. To determine the location of the pharmacological sites on OS_2 and explain the large difference in neurotoxicity between OS_2 and the structurally-related OS_1, we prepared large chimeras between the two proteins by grafting the divergent N-terminal, central and C-terminal regions of OS_1 onto the OS_2 structure.

EXPERIMENTAL PROCEDURES

Construction of the OS_2 synthetic gene and mutants

A number of considerations have been taken into account for the production of OS_2. First, as we could not obtain mRNA from the venom gland of the Australian Taipan snake, we constructed a synthetic gene for OS_2 based on the sequence of the venom protein (52). Second, since our experience indicates that some $sPLA_2$s cannot be easily refolded when produced in *E. coli* while they can be obtained in a native form from insect cell systems and *vice-versa* (11,31), we tried expression of OS_2 in both S2 *Drosophila* cells and *E. coli*. Finally, since we found that the use of *E. coli* preferred codons is not critical when the $sPLA_2$ is fused to the N-terminal sequence of proteins such as glutathione S-transferase or thioredoxin (unpublished data and (53)), we designed the OS_2 synthetic gene using *Drosophila* preferential codons (54) that should allow expression in both insect cells and *E. coli*. The synthetic cDNA sequence (available on request) was designed using the backtranslate program from the Genetic Computer Group and assembled from three sets of six partially overlapping oligonucleotides

(40-mer) in a three-step PCR process using Dynazyme polymerase (Finnzyme laboratories). In the first round of PCR (final volume 20 µl), each set of oligonucleotides (5 µg each, 3 forward and 3 reverse, 20-mer complementary sequences) was annealed (60 °C/20 s), elongated (72 °C/20 s) and denaturated (94 °C/20 s) for 25 cycles. The DNA assemblies were further amplified in a second round of PCR (25 cycles, each 94 °C/20 s, 60 °C/20 s, 72 °C/20 s) using the two external oligonucleotides. The three PCR products were purified on agarose gel and used for a last round of PCR using the two oligonucleotides flanking the full OS_2 synthetic gene. The final PCR product was tailed with *Taq* polymerase, subcloned into the pGEM-T easy vector (Promega Corp.) and sequenced. The OS_2 cDNA was subcloned in frame into the S2 expression vector harboring the human group IIA $sPLA_2$ signal peptide or into the pAB_3 vector (11). Point mutants were prepared in a two-step PCR standard procedure (55) using complementary primers containing the mutation and primers flanking the OS_2 synthetic gene inserted into the pAB_3-OS_2 vector. Chimeras were constructed by inserting by PCR synthetic DNA fragments coding for the N-terminal, central and C-terminal regions of OS_1 (Fig. 1) into the pAB_3-OS_2 construction. All the final PCR products were entirely sequenced.

Production of recombinant OS_2 mutants and chimeras in Drosophila S2 cells and E. coli

Initial attempts to express OS_2 in *Drosophila* S2 cells were unsuccessful, although we used a protocol similar to the one used for several mouse and human $sPLA_2$s (11). Conversely, wild-type (WT) OS_2, single point mutants and chimeras could be produced in *E. coli* using the pAB_3 vector (11). The $sPLA_2$s were produced as insoluble C-terminal fusion proteins with the truncated glutathione S-transferase (8.7 kDa) and the factor Xa/trypsin proteolytic site (Ile-Glu-Gly-Arg) located before the N-terminal residue of the mature $sPLA_2$. Expression of fusion proteins as inclusion bodies in *E. coli* BL21 cells and sulfonation from 100 mg of protein were performed as described (56). For refolding, the sulfonated protein was dissolved in 500 ml of 6 M guanidine-HCl, 50 mM Tris-HCl pH 8.0 and dialyzed (8 kDa membranes, Spectrum laboratories) against 8 liters of 0.7 M guanidine-HCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA and 5 mM L-cysteine (5 mM L-methionine was added for the central and C-terminal chimeras to prevent methionine oxidation) for 48 h at 4 °C. For the N-terminal chimera the sulfonated protein was resuspended in 500 ml of 8 M urea, 0.1 M NH_4Cl, 50 mM Tris-HCl pH 8.0 and refolded by dialysis against 8 liters of 1.6 M urea, 0.1 M NH_4Cl, 50 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$ and 5 mM L-cysteine for 48 h at 4 °C. The refolded protein was then dialysed against 8 liters of 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$ and clarified by centrifugation (10,000 g, 30 min). The protein solution was subjected to trypsin digestion (1:30 trypsin:protein) which was monitored online by *E. coli* enzymatic assays (11) and mass spectrometry, and the reaction was stopped by adding 1% acetic acid and 1 mM phenylmethylsulfonyl fluoride. The digested protein was concentrated to 20 ml by ultrafiltration using an Amicon stirred cell concentrator with a YM10 membrane, and the buffer was exchanged to 1% acetic acid/10% acetonitrile (ACN). The refolded protein was purified sequentially by cation exchange HPLC on a Spherogel TSK SP-5PW column (3.3 ml, 10 µm, 7.5 × 75 mm, Tosoh Biosep, 1 ml/min, linear gradient of ammonium acetate (0 to 1 M, pH 6.8 over 50 min) in 10% ACN) followed by C18 reverse phase HPLC (19.3 ml, 10 × 250 mm, 100 Å, 5 µm, Beckman, 4 ml/min, linear gradient of ACN in 0.1% TFA (10–28% ACN over 18 min, 28–35% ACN over 42 min). The lyophilized proteins were quantified by OD_{280nm} using their calculated molar absorption coefficients, and analyzed by MALDI-TOF mass spectrometry, SDS-polyacrylamide gel electrophoresis, and circular dichroism (CD). MALDI-TOF analysis of $sPLA_2$s was carried out on an Applied Biosystems voyager DE-PRO mass spectrometer in linear mode with sinapinic acid as a matrix, using external calibration. The secondary structure of all proteins was evaluated by CD using a Jasco PTC-423S (J-810) apparatus and analyzed with the program CDNN (CD Spectra deconvolution, version 2.1). Proteins were diluted to 0.1 or 0.2 mg/ml in 10 mM Na^+-borate pH 8.0, 0.1 mM EDTA and spectra were recorded at 20 °C with a scanning speed of 20 nm/min between 190 and 260 nm. The final CD spectrum was obtained from the average of 15 runs.

Enzymatic activity and interfacial binding to phospholipid vesicles

E. coli enzymatic assays were performed using [^{3}H]oleate-radiolabeled membranes (11). The headgroup substrate specificity was measured on large unilamellar vesicles of pure POPG, POPS, or POPC with the fatty acid-binding protein assay as described (11). The initial velocities were measured at 37 °C in 1.3 ml of Hanks' balanced salt solution containing 1.3 mM Ca^{2+}, 0.9 mM Mg^{2+}, 30 μM phospholipid extruded vesicles, 1 μM 11-dansyl-undecanoic acid, and 10 μg of rat liver fatty acid binding protein. Excitation was at 350 nm and emission at 500 nm. Assays were calibrated by adding known amounts of oleic acid and measuring the decrease in fluorescence. Reactions were started by adding $sPLA_2$. Binding studies of $sPLA_2$s to sucrose-loaded, 0.1 μm unilamellar vesicles of diether phospholipids (20 mole% DOetPS/80 mole% DOetPC) were performed as described previously (11). $sPLA_2$ binding studies were carried out in 5 mM MOPS, pH 7.4, 0.1 M KCl, 2 mM $CaCl_2$ at room temperature using the centrifugation method in which the amount of enzyme remaining in the supernatant above pelleted vesicles is quantified by enzymatic assays (for high specific activity enzymes) or silver-stained 16% SDS-PAGE gels (for both low and high specific activity enzymes). Assays were made in the presence of 0.5 μg of $sPLA_2$ and different concentrations of phospholipid vesicles in a total volume of 100 μl to measure the affinity (K_d) of the $sPLA_2$ for the phospholipid vesicle.

Receptor binding assays and cross-linking studies

Competition binding assays between iodinated OS_2 and unlabeled OS_2 mutants on N-type and M-type receptors were performed under equilibrium binding conditions as described (20,[30], 31), except that OS_2 was labeled to a specific activity of 3000–3500 cpm/fmol using lactoperoxidase as follows. The iodination was performed in a total volume of 60 μl of 0.1 M NaH_2PO_4 pH 6.5 containing 0.5 nmole OS_2, 0.5 nmole ^{125}I-Na (Perkin Elmer), 0.5 μg lactoperoxidase (Sigma L8257), and 20 μl of hydrogen peroxide (Sigma H1009) diluted 1/40,000 in H_2O. The reaction was initiated by adding 10 μl hydrogen peroxide at 0 and 10 min. After incubation at room temperature for 20 min, the mixture was diluted to 1 ml with H_2O/ ACN/TFA (90/10/0.1) and applied to a C18 end-capped reverse phase HPLC column (Merck purospher STAR, 55 × 4 mm, 3 μm, 100 Å, bed volume 0.7 ml). Elution was performed at 1 ml/min using a linear gradient of ACN in 0.1% TFA (10–25% ACN over 10 min, 25–50% ACN over 50 min). Fractions containing active iodinated OS_2 were identified by binding assays on M- and N-type receptors. Binding on M-type receptors was carried out on membranes from HEK 293 cells (ATCC CRL-1573) transfected with either rabbit or mouse cloned M-type receptors (31,57). Binding on N-type receptors was carried out on mouse brain membranes (30) or HeLa P4 cell membranes (20). Briefly, membranes, ^{125}I-OS_2 and unlabeled $sPLA_2$s were incubated at room temperature in 0.5 ml of binding buffer (140 mM NaCl, 0.1 mM $CaCl_2$, 20 mM Tris-HCl pH 7.4 and 0.1% bovine serum albumin). Incubations were started by addition of membranes and filtered after 1 h through GF/C glass fiber filters pre-soaked in 0.5% polyethyleneimine, 10 mM Tris-HCl pH 7.4. Iodination and binding experiments with the C-terminal chimera were performed as for WT OS_2. Cross-linking experiments on mouse brain membranes were performed as described (30) using 200 μM of suberic acid bis-*N*-hydroxysuccinimide ester (DSS, Sigma).

Central neurotoxicity

Central neurotoxicity was evaluated by measuring the minimal lethal doses (LD_{100}) after intracerebroventricular injection of $sPLA_2$s (5 μl, dissolved in 0.9% NaCl) into BALB/c male mice (42–45 days old, 18–20 g) (58). LD_{100} is defined as the lowest amount of $sPLA_2$ that kills all mice (n≥2), half of this dose being not lethal 4 h after injection (n≥2). All procedures were performed in accordance with the European Commission Council Directives.

Chick isolated biventer cervicis muscle assays

Biventer cervicis muscles were removed from male chicks (4–10 days-old), mounted in 5 ml organ baths and maintained at 34 °C under 1 g resting tension in Krebs solution of the following composition (mM): NaCl 118.4; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.0; glucose 11.1; $CaCl_2$ 2.5, bubbled continuously with 95% O_2 and 5% CO_2. Isometric contractions were measured via a Grass transducer (FT03) connected to a Maclab/8 system. Twitches were evoked by stimulating the motor nerve (supramaximal voltage, 0.2 ms, 0.1 Hz) via silver electrodes connected to a Grass S88 stimulator. Nerve mediated (indirectly evoked) twitches were confirmed by abolition of twitches after addition of 10 µM (+)-tubocurarine (Sigma). In the absence of electrical stimulation, responses (directly evoked) to exogenous acetylcholine (ACh, Sigma, 1 mM, 30s), carbachol (CCh, 20 mM, 60 s) and potassium chloride (KCl, 40 mM, 30 s) were obtained prior to the addition of $sPLA_2$ and at the end of the experiment. This allows the classification of the $sPLA_2$ effects observed in the chick preparation into presynaptic or postsynaptic actions. Preparations were allowed to equilibrate for at least 30 min with continuous stimulation before addition of $sPLA_2$.

Myotoxic effects on mice

$sPLA_2$s were dissolved in phosphate-buffered saline (PBS : 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2). Aliquots of 100 µl, containing 5 µg of $sPLA_2$, were injected intramuscularly in the right thigh of CD-1 mice (18–20 g). Control mice received 100 µl of PBS under otherwise identical conditions. Three hours after injection, a blood sample obtained by cutting the tip of the tail was collected into heparinized capillary tubes and centrifuged. Plasma creatine kinase activity was determined using the Sigma kit No. 47-UV (Sigma-Aldrich). Creatine kinase activity is expressed in units U/l, where one unit is defined as the activity of the enzyme that produces 1 µmol of NADH/min under the conditions of the assay. Mice were observed for 8 h for symptoms of systemic toxicity. Statistical analysis was performed by determining the significance of the differences of the means of all experimental groups by ANOVA. Comparisons between pairs of means were performed by the Tukey Kramer method.

Anti-HIV-1 activity

Single rounds of HIV-1_{BRU} virus infection in HeLa P4 cells were performed as described previously (20). Briefly, HeLa P4 cells were seeded into 24-well plates (80,000 cells per well) and infected the next day with 100 µl of HIV-1_{BRU} viral supernatant (100 ng of Gag p24). Virus and cells were left in contact for 8 h at 37 °C in the absence or presence of the different $sPLA_2$s, and the culture medium was then replaced with fresh medium containing 50 µM 3'-azido-3'-deoxythymidine (AZT, Sigma) to prevent for another round of infection. Two days after infection, HeLa P4 cells were lysed and β-galactosidase activity was measured as an index of virus infection.

Antimalarial activity

Inhibitory effect toward *P. falciparum* intraerythrocytic development was assayed as previously described (21) in complete medium containing either 8% heat-inactivated human serum or 0.5% AlbuMAX II, a culture medium supplement based on calf serum lipid-enriched albumin (InVitroGen). Various concentrations of each $sPLA_2$ (100 µl/well) were distributed in a 96-well plate, then 100 µl of a *P. falciparum* culture (1.5% parasitemia and 4% hematocrit) was added. After 24 h of incubation, 0.5 µCi ^{3}H-hypoxanthine (ICN Biomedicals) was added per well, and parasites were grown for an additional 24 h. Plates were freeze-thawed and harvested on filters. Dried filters were moistened in scintillation liquid mixture (OptiScint, Hisafe) and counted in a 1450 Microbeta counter (Wallac, Perkin Elmer).

RESULTS

Rationale for the design and recombinant production of wild-type OS_2, single point mutants and chimeras

Because the production of recombinant $sPLA_2$s can be problematic (11), we initially tried to express WT OS_2 from a synthetic gene in *Drosophila* S2 cells and *E. coli* using the pAB_3 expression vector system (see methods). Only the expression in *E. coli* was successful. Two different site-directed mutagenesis approaches were then developed to investigate the structure-function relationships of OS_2. First, we prepared single point mutants of OS_2 (glycine-30 to serine, histidine-48 to glutamine and aspartate-49 to lysine) with the aim to abolish its enzymatic activity, but not its biological effects and/or binding properties to receptors (Fig. 1 and Fig. 2). These three residues are absolutely conserved in all active $sPLA_2$s, are located in the active site or Ca^{2+}-binding loop, and are known to be essential to catalysis (51). Moreover, venom $sPLA_2$s that harbor similar mutations occur naturally, are catalytically inactive, yet they can exert diverse pharmacological effects (59–61). Mutation of Histidine-48 by glutamine has been shown to dramatically reduce enzymatic activity (62,63). Mutations of glycine-30 and aspartate-49 are known to affect the binding of substrate and Ca^{2+}, an essential cofactor for $sPLA_2$ activity and substrate binding (60,[64],65). All three mutations were thus expected to produce catalytically inactive mutants of OS_2 for distinct reasons. We also constructed the OS_2 double mutant K31L-R34S to analyze the role of amino acids at positions 31 and 34, which are hypervariable within the highly conserved Ca^{2+}-binding loop of $sPLA_2$s (66). Lysine-31 and arginine-34 of OS_2 were respectively replaced by leucine and serine which are found in the non toxic porcine pancreatic $sPLA_2$ (52).

In the second approach, we designed chimeras between OS_2 and OS_1 in an effort to identify the main regions of OS_2 which are responsible for its molecular properties and biological effects. OS_1 was also purified from Taipan snake venom and is structurally homologous to OS_2, but has quite distinct biological properties (30,[52],58). Pharmacologically, OS_1 binds only very weakly to N-type receptors, has a lower specific activity on phospholipid substrates (see below), and is neither neurotoxic, nor myotoxic, nor able to potently inhibit HIV-1 replication ((20,30) and below). However, both OS_1 and OS_2 are high affinity ligands for the M-type receptor (58), and both also have antimalarial activity (see below). Structurally, OS_1 is 54% identical to OS_2 (Fig. 1B). The level of identity is very high in the active site and Ca^{2+}-binding loop, while it is much lower in the N-terminal, central, and C-terminal regions (Fig. 1B). A three-dimensional model of OS_2 was produced by homology modeling using the x-ray structure of notexin as a template (Fig. 2). A model for OS_1 (not shown) was also produced using as template the three-dimensional structure of the acidic $sPLA_2$ from *Ophiophagus hannah* venom (pdb structure 1gp7). Based on the major structural differences between the two proteins, we constructed three different chimeras (Fig. 1 and Fig. 2). Importantly, all the residues in the protein segments that were exchanged in the chimera constructs and that point toward the core of the protein are identical between OS_1 and OS_2 except for the conservative change of Met-8 in OS_2 being replaced with Leu in OS_1. Thus, all but one of the residue changes occur at the molecular surface, and segment exchanges should not cause an adverse structural alteration of the chimeric proteins.

Recombinant expression and structural characterization of OS_2, single point mutants and chimeras in E. coli

All OS_2 proteins could be produced from *E. coli* with high yields (Table 1). The HPLC-purified proteins migrated as single bands of the expected size on a Laemmli SDS gel (Fig. 3A). For all $sPLA_2$s, the molecular masses measured by MALDI-TOF mass spectrometry matchedthe calculated masses within less than 1 Da (Table 1), clearly establishing that all the disulfide bonds were formed in the proteins, and that residue side chains had not been chemically oxidized or further modified during the refolding and purification processes. We also compared the overall conformation and secondary structure of venom, WT recombinant OS_2, and its mutants by CD spectroscopy. The CD spectra were very similar for all proteins, including the three chimeras (Fig. 3B and not shown). Taken together, these results indicate that the recombinant proteins are pure and properly folded with all their disulfide bonds appropriately formed.

Lethal potency of OS_2 and its mutants in mice

Both venom and recombinant WT OS_2 are highly neurotoxic to mice following intracerebroventricular injection with an identical LD_{100} of 2 µg/kg (Table 2). Interestingly, the active site mutant H48Q is still neurotoxic (33 µg/kg), although the LD_{100} is 16-fold lower than that of WT OS_2. Conversely, the two other active site mutants G30S and D49K have very low or no toxicity with LD_{100} values of 3,330 µg/kg or higher (Table 2). The double mutant K31L-R34S appears to be nearly as toxic as WT OS_2, suggesting that positions 31 and 34 are not critical, at least when mutated into leucine and serine. In marked contrast with WT OS_2, OS_1 displays a very low toxicity with an LD_{100} of 3,500 µg/kg. The N-terminal chimera is not toxic at a concentration as high as 1,500 µg/kg, and thus behaves similarly to OS_1. However, the central and C-terminal chimeras retain high toxicities, with LD_{100} values of 70 and 33 µg/kg, respectively.

Enzymatic properties of OS_2 and its mutants

The enzymatic properties of WT recombinant OS_2 were found to be identical to those of OS_2 purified from venom (Table 3). WT OS_2 shows a high specific activity on *E. coli* membranes and is more efficient on the anionic phospholipid POPG than on the anionic phospholipid POPS or the zwitterionic phospholipid POPC (Table 3). As expected, mutations of G30, H48 and D49 in the Ca^{2+}-loop and active site of OS_2 dramatically affected the catalytic activity, but did not alter the headgroup substrate specificity. Indeed, the specific activities of H48Q and G30S mutants were both reduced by about 500-fold. The activity of OS_2 D49K was reduced to undetectable levels. The mutations K31L and R34S in the Ca^{2+}-loop did not affect the specific activity and only slightly modified the headgroup substrate specificity of OS_2.

In the case of the OS_2/OS_1 chimeras, a strong decrease in specific activity was observed for the N-terminal (1,000-fold) and C-terminal (500-fold) chimeras (Table 3). Conversely, the central chimera retains a high specific activity, which can be up to 5-fold higher than that of WT OS_2. Some changes in the headgroup substrate specificities were observed for the central chimera and the C-terminal chimera, which appeared to have a substrate specificity more similar to that of OS_1.

We also measured the binding properties of the different $sPLA_2$s to diether phospholipid vesicles made up of 20 mole% DOetPS/ 80 mole% DOetPC. WT OS_2 binds much more tightly to these phospholipid vesicles than OS_1 (Table 3). The double mutant K31L/R34S and the active site mutant H48Q also bind tightly, while G30S and D49K do not bind at all under our binding assay conditions. Among the three chimeras, the central chimera binds as tightly as WT OS_2, while the two other chimeras do not bind. When the binding of WT OS_2 was measured in the absence of free Ca^{2+} (2 mM EDTA instead of 2 mM $CaCl_2$ in binding buffer, see methods), the $sPLA_2$ was completely unable to bind to vesicles (not shown).

Binding of OS_2 and its mutants to M-type and N-type receptors measured by competition binding assays with iodinated OS_2

Binding studies on mouse and rabbit M-type receptor show that most mutants bind to this receptor with $K_{0.5}$ values similar to that of WT OS_2 (Fig. 4A and Table 4). The only exceptions were the N-terminal and central chimeras which show 4- to 30-fold lower affinities. The fact that all mutants including chimeras bind to the M-type receptor with high affinities further indicates that all of them are properly folded. Finally, the double mutant K31L/R34S was constructed to evaluate the contribution of residues 31 and 34 to M-type receptor binding. Indeed, we previously found that mutation of Leu-31 in the pancreatic $sPLA_2$ decreased its affinity for the receptor, suggesting that this position is important (52). We also noticed that the positions 31 and 34 are the only two hypervariable positions within the conserved Ca^{2+}-loop, suggesting that the nature of the residues at these positions may determine, at least in part, the different binding properties of $sPLA_2$s to the receptor (52). In the OS_2 double mutant K31L/R34S, the OS_2 residues have been replaced by those of the pancreatic $sPLA_2$ with the goal to decrease the binding affinity. The fact that no decrease was observed suggest that the positions 31 and 34 are not crucial for the OS_2 interaction, or that these mutations can be accomodated in the OS_2 structure to maintain a high affinity. Further work including co-crystallization of the $sPLA_2$-receptor complex is required to address the contribution of residues at positions 31 and 34 in the $sPLA_2$-M-type receptor interaction.

We next investigated the binding properties of the different mutants to N-type receptors present in mouse brain membranes and human HeLa P4 cells. These two sources of N-type receptors were chosen because of their potential implication in two of the pharmacological models used in this study, *i.e.* neurotoxicity in mouse brain and inhibition of HIV replication. Binding to N-type receptors was found to be sensitive to the different mutations analyzed, but to a different extent (Figs. 4B and 4C, and Table 4). The mutant K31L-R34S retained an affinity close to that of WT OS_2 for both mouse brain and HeLa P4 N-type receptors. The mutant H48Q was also able to efficiently bind to the N-type receptors expressed in mouse brain and HeLa P4 cells, yet its affinity was 50-fold lower than that of WT OS_2. D49K and the C-terminal chimera also bind to the N-type receptors with nanomolar affinities, even though their affinities are 180-to 1,230-fold lower than for WT OS_2. Mutation of glycine-30 produces a very dramatic effect, decreasing the affinity by a factor of about 20,000. Finally, the N-terminal and central chimeras were unable to compete with iodinated OS_2 for binding to mouse brain and Hela P4 cell membranes.

Binding of OS_2 and its mutants as revealed by cross-linking of labeled OS_2 to mouse brain membranes

Because some of the above binding results did not correlate well with the central neurotoxic properties of OS_2 mutants (Table 2), while a relationship between $sPLA_2$ neurotoxicity and N-type receptor binding was previously proposed (30,67), we analyzed the binding properties of the OS_2 mutants by cross-linking experiments of radiolabeled OS_2 to mouse brain membranes (Fig. 5). These experiments constituted another method to visualize the ability of the OS_2 mutants to bind to N-type receptor proteins. When ^{125}I-OS_2 was allowed to bind to mouse brain membranes and subsequently cross-linked with DSS, several proteins of 14, 18, 39 and 50 kDa were labeled (Fig. 5). The cross-linking of these proteins is specific because it is fully prevented by an excess of unlabeled OS_2 (300 nM), but not OS_1, which is unable to bind to N-type receptors (Table 4). In agreement with the competition binding assays (Table 4), the mutant K31L-R34S completely prevented the cross-linking of ^{125}I-OS_2 to the N-type receptors. Similarly, the mutants H48Q and D49K decreased the labeling, whereas the mutant G30S and the N-terminal chimera had no effect. However, the central and C-terminal chimeras appeared more potent than expected from the competition assays (Fig. 4), especially for the C-terminal chimera which efficiently prevented the cross-linking of iodinated OS_2.

Binding properties of iodinated C-terminal chimera

To reconcile the above results, we postulated that WT OS_2 binds with high affinities to at least two different populations of binding sites in mouse brain, while the central and C-terminal chimeras bind with high affinity to only a subset of these receptors. This receptor subset would correspond to those visualized by cross-linking experiments (Fig. 5). According to this hypothesis, the WT OS_2-specific population(s) of binding sites (which could be proteins and/or phospholipids) should be abundant in mouse brain membranes and responsible for most of the signal measured with iodinated WT OS_2 in competition binding assays (Fig. 4). Additionally, the two chimeras should have weak affinity for these binding sites, and thus they should not compete efficiently (Fig. 4).

To investigate this hypothesis, we iodinated the central and C-terminal chimeras to perform direct binding studies and identify a subset of OS2 binding sites. Binding assays with the central chimera could not be done as it became highly oxidized during the iodination procedure with lactoperoxidase (data not shown), even when iodination was performed under very mild conditions (68). Conversely, the C-terminal chimera could be iodinated without oxidation and used in direct binding experiments. Fig. 6 shows a comparison of the binding properties of iodinated WT OS_2 and C-terminal chimera obtained in parallel experiments on the same mouse brain membrane preparations. Fig. 6A and B show that the binding of both proteins to mouse brain membranes is saturable and specific. In both cases, the Scatchard plots of the specific binding are best fitted by curvilinear curves, suggesting that the two ligands bind to at least two distinct families of binding sites with very high affinities in the pM range (Fig. 6C and D). However, the total number of binding sites for the chimera (0.042 pmol/mg of total protein) appears to be about 7-fold lower than that for WT OS_2 (0.3 pmol/mg of total protein). This result plus the fact that WT OS_2 competes with the labeled chimera for binding (Fig. 6F) indicates that the chimera indeed binds to a minor subset of WT OS_2 binding sites. Additionally, when the competition binding assays for the different chimeras were performed using the iodinated C-terminal chimera as labeled ligand, the affinities were much higher than those obtained using labeled WT OS_2 (Fig. 6E and F). Together, the binding properties of the labeled C-terminal chimera support the hypothesis of at least two major populations of OS_2 binding sites, among which one binds with high affinity the central and C-terminal chimeras and has molecular masses corresponding to those revealed by the cross-linking experiments (Fig. 5). The curvilinear curve obtained with the iodinated C-terminal chimera suggests that the binding sites recognized by WT OS_2 and the C-terminal chimera are still complex in nature and may be represented by several independent targets.

Effects of OS_2 and its mutants on neuromuscular transmission

Neurotoxic snake venom $sPLA_2$s are called β-neurotoxins because they affect neuronal or neuromuscular transmission by acting presynaptically, even though some of them can also exert postsynaptic and myotoxic effects (2,[14],69). OS_2 is lethal when injected intra-cerebroventricularly into rat or mouse brain (30,[48],70), and has been shown to block acetylcholine release in a neuro-neuronal synapse in *Aplysia* (49), suggesting that OS_2 is also a β-neurotoxin. To confirm that OS_2 acts presynaptically at a vertebrate neuromuscular junction, we analyzed its effects and those of its mutants on the chick *biventer cervicis* neuromuscular preparation (Fig. 7). Only WT OS_2 and the K31L-R34S mutant, when tested at 200 nM, were able to reduce the height of indirect twitches evoked by electrical nerve stimulation (Fig. 7A). At lower concentrations of 20 and 50 nM, WT OS_2 had no twitch reduction effect, indicating that OS_2 is not very potent on this neuromuscular preparation. In line with this rather low potency, all the other mutants and chimeras were found to be inactive when assayed at 200 nM. We next analyzed the effect of WT OS_2 on contractions of the chick *biventer cervicis* preparation directly evoked by various agonists including acetylcholine, carbachol and potassium chloride (Fig. 7B). No significant inhibition of the contractions was observed in the presence of WT OS_2 (Fig. 7B) or mutants (not shown) at 200 nM.

Myotoxic effects of OS_2 and its mutants

We also analyzed whether OS_2 can exert myotoxic effects when injected intramuscularly into mice (Fig. 8). Only the groups of mice injected with recombinant WT OS_2 and the mutant K31L-R34S showed signs of myotoxic intoxication, as measured by plasma creatine kinase activity. In both cases, mice also developed signs of neurotoxicity, with paralysis of the limbs and respiratory difficulties. Three out of the five mice injected with recombinant WT OS_2 and the mutant K31L-R34S died within 8 hr. In addition, mice in these two experimental groups showed macroscopic swelling and immobilization of the injected limb consistent with myotoxic effects, as previously observed for other myotoxins (71). In contrast, mice injected with the other mutants, chimeras, and venom OS_1 did not show any evidence of intoxication, nor local myotoxic effects at the site of injection.

Anti-Plasmodium activity of OS_2 and its mutants

WT OS_2 and its mutants were tested for their ability to inhibit the intra-erythrocytic growth of *P. falciparum*, the causative agent of the most severe form of human malaria (Table 2). The effect of $sPLA_2$s was assayed in the presence of human serum (8%) or in a semi-defined medium (0.5% AlbuMAX II, a serum substitute devoid of lipoproteins) to evaluate whether $sPLA_2$s act directly or indirectly by modification of serum components including hydrolysis of lipoprotein phospholipids (72). In the presence of serum, both OS_1 and WT OS_2 inhibited *P. falciparum* growth. Remarkably, OS_1 was even more potent than OS_2 (Table 2). The mutants H48Q, D49K, G30S and the N-terminal chimera, that display extremely low enzymatic activities on all types of phospholipids (Table 3), were totally inactive against *P. falciparum*. Conversely, the mutant K31L/R34S, the central chimera, and to a lesser extent the C-terminal chimera, were still able to inhibit the growth of the parasite. The results were dramatically different in the presence of AlbuMAX, where most of the $sPLA_2$s were inactive. Under those conditions, only the $sPLA_2$s which are extremely potent in the presence of human serum were still capable of preventing the growth of the parasite, yet at much higher concentrations (Table 2).

Inhibition of HIV replication by OS_2 and its mutants

We previously found that several but not all venom $sPLA_2$s can block the entry of HIV-1 into host cells (20). Venom and recombinant WT OS_2 as well as the mutant K31L-R34S were found to be inhibitors of HIV-1 infectivity with IC_{50} values in the nM range (Table 2). Conversely, at concentrations up to 1 μM, the mutants H48Q, D49K, G30S and the N-terminal chimera did not inhibit HIV-1 infection. However, the central and C-terminal chimeras still retained a fairly potent antiviral activity, with IC_{50} values between those of WT OS_2 and OS_1 (Table 2).

DISCUSSION

A large number of studies using *in silico* protein sequence comparisons, chemical modification of amino acids, and more recently site-directed mutagenesis on different toxic $sPLA_2$s have been carried out over the past 30 years (23,[35],43). However, the results from these studies have remained difficult to interpret for several reasons: i) $sPLA_2$s are quite compact molecules and some mutations may cause structural changes at sites remote from the mutation site; ii) they probably exert most of their biological properties through relatively large molecular surfaces encompassing several discrete domains (*i.e.* the N-terminal helix, Ca^{2+}-loop, *etc.*.); iii) their mechanism of action may include several steps (39,40); iv) several studies have been based on chemical modification or mutagenesis of single amino acids, which in most cases have led to only relatively modest effects; v) the results obtained for a particular venom $sPLA_2$ may not apply to other $sPLA_2$s; vi) some amino acids that are important for catalytic activity may also be involved in receptor binding or biological activities.

The main goal of our study was to obtain new insights into the structure-function relationships of venom $sPLA_2$s using OS_2 from the Australian Taipan snake venom as a template, and with a particular focus on its central neurotoxic effects. Our first approach employed site-directed mutagenesis at the active site which, to our knowledge, has never been used to establish the relationship between enzymatic catalysis and central neurotoxicity. The second approach used large chimeras between OS_1 and OS_2, and is based on the observation that the two proteins from the same Taipan venom display very distinct catalytic, toxic, and N-type receptor binding properties. The aim of this second approach was to produce very dramatic changes in the molecular and pharmacological properties of OS_2, and thus map unambiguously which of the three regions plays the major role. A similar chimeric approach was used for ammodytoxins, but in that case, the predicted C-terminal neurotoxic region of ammodytoxin A was introduced into the non toxic ammodytoxin I2, and only mild effects were observed (34). As discussed below in more details, our results clearly indicate that : i) based on the clear dissociation observed for the H48Q mutant and the C-terminal chimera, the catalytic activity of OS_2 is responsible for little, if any, of the central neurotoxicity; ii) the N-terminal region of OS_2 and some well conserved residues of the Ca^{2+}-loop, but not the central and C-terminal regions, are key elements for central neurotoxicity and several other pharmacological effects; iii) The same N-terminal region allows OS_2 to bind to a subset of N-type receptors which is likely to be associated with central neurotoxicity; iv) The antimalarial effect of OS_2 and OS_1 appears to be dependent on the catalytic activity of these enzymes on serum lipoproteins. Together, these findings support the view that a single $sPLA_2$ exerts its different pharmacological effects through distinct mechanisms that depend or not on its enzymatic activity (2).

Enzymatic properties of the OS_2 mutants and chimeras

Our two approaches have generated OS_2 mutants which have dramatically lower enzymatic activity for different reasons. The single point mutants G30S, H48Q and D49K have $sPLA_2$ activity reduced by 500-fold or more, essentially because they cannot efficiently bind or hydrolyze phospholipids in their active site (51). Conversely, the N-terminal and C-terminal chimeras have reduced activity because they cannot efficiently bind to the phospholipid membrane interface. Indeed, the $sPLA_2$ catalysis consists of two distinct steps, in which the enzyme first binds to a surface of about 40–50 phospholipids at the phospholipid-water interface (interfacial binding) and then forms a Michaelis-Menten complex by binding a single phospholipid molecule into the active site (51). These two steps involve two topologically distinct $sPLA_2$ domains, *ie* the interfacial binding surface and the active site slot. The active site contains six residues which are conserved in all active $sPLA_2$s among which G30, H48 and D49 play major catalytic roles. Conversely, the interfacial binding surface comprises at least 10–15 residues which are not conserved between $sPLA_2$s and which can be located in the N-terminal, central and C-terminal regions of $sPLA_2$s. The nature of these residues will determine the affinity of the enzyme for different lipid interfaces. Over the years, it has become clear that the interfacial binding of $sPLA_2$s is the key step that governs and explains most of the differences in specific activities on different phospholipid substrates. From a large number of studies, first performed on venom $sPLA_2$s and then on the various mammalian enzymes, it is now clear that while basic, neutral, and aliphatic residues found in the interfacial binding domain contribute to phospholipid binding, a few aromatic residues, especially tryptophans, play major roles in $sPLA_2$ binding, particularly to zwitterionic phospholipids (73–81).

The 500- to 1,000-fold drop in catalytic activity of the N-terminal and C-terminal chimeras is largely explained by their decreased binding affinity for phospholipid vesicles (Table 3). This indicates that the N-terminal and C-terminal regions of OS_2, but not the central one, contain key residues which are involved in high affinity interfacial binding and are not present in OS_1. Among these residues, OS_2 has a tryptophan located in the C-terminal region (Fig. 2 and position 117 in Fig. 1) that would explain at least in part why the C-terminal chimera is less active than WT OS_2 (Table 3). Accordingly, mutation of asparagine to a tryptophan at this position (N117W) in the pancreatic group IB $sPLA_2$ results in a 18-fold increase in interfacial binding (78). OS_2 has a second tryptophan at position 75 (Fig. 1), but this residue is located on the β-wing structure in the central region, far away from the interfacial binding domain, and thus most probably does not participate in interfacial binding. This is consistent with the fact that for many $sPLA_2$s, the critical interfacial binding residues are present in the N-terminal and C-terminal regions (79,[80],[82]–86). The slightly altered enzymatic activity and substrate specificity of the central chimera (Table 3) may be related at least in part to the deletion of the OS_1 pancreatic loop, whose deletion in the pancreatic group IB $sPLA_2$ produced similar effects (87).

The H48Q mutant does not show altered interfacial binding under our experimental conditions, indicating that the drop in activity is essentially due to its inability to hydrolyze phospholipids in the active site. Conversely, the very low enzymatic activity of the OS_2 active site mutants G30S and D49K is due not only to their inability to bind the substrate at the active site, but also to altered interfacial binding (Table 3). Previous studies on the pancreatic group IB and human group IIA $sPLA_2$s have shown that the mutations G30S, H48Q and D49K largely alter phospholipid catalysis, but have little effects on interfacial binding (62–[65],88). Similar conclusions were drawn from enzymatic studies and crystal structures of venom $sPLA_2$s harboring the natural mutations G30S, H48Q and D49K (59–[61],89). However, detailed studies on the role of the Ca^{2+} cofactor in catalysis by pancreatic and bee venom $sPLA_2$s indicated that the interfacial binding and the binding at the active site are interconnected, where the Ca^{2+}-dependent binding of the substrate at the active site partially drives the interfacial binding of $sPLA_2$ by mass action (81,[90],91). Under our phospholipid vesicle assay conditions (20 mole % DOetPS/ 80 mole% DOetPC), the mutants G30S and D49K do not bind efficiently to the vesicles, which can be explained at least in part by the fact that they cannot bind Ca^{2+} and thus their substrate at the active site. This result also agrees with the absence of vesicle binding for WT OS_2 when Ca^{2+} is omitted (not shown). Additionally, the structural data accumulated so far also suggest that the mutations G30S, H48Q and D49K locally alter to different extents the conformations of the Ca^{2+}-loop and N-terminal helix, with conformational changes induced by the mutation H48Q being smaller than those induced by mutation of Asp-49 (61,[63],[92],93). On the other hand, deletion of the C-terminal region of the pancreatic $sPLA_2$ affects substrate binding at the active site (90). In the case of His-48 alkylation, an allosteric coupling between the catalytic site and the membrane binding surface of the $sPLA_2$ has been proposed (43–[46], 94). Furthermore, the hydrogen bonding network connecting the active site to the interfacial binding domain and which involves His-48 is likely to be important for the conformational stability of $sPLA_2$s (46,92). Finally, a recent study showed that binding of an hydrophobic ligand in the active site of a Lys-49 venom $sPLA_2$ induces conformational changes in the C-terminal domain (95). In summary, it is likely that the drop in activity observed in the case of the OS_2 active site mutants (Table 3) is due in large part to the point mutation affecting a key catalytic functionality, *ie* phospholipid hydrolysis for the H48Q and Ca^{2+}-dependent phospholipid binding at the active site for G30S and D49K. However, we propose that these mutations may also induce small (H48Q) or significant (G30S and D49K) conformational changes at the surface of the protein, especially on the N-terminal alpha-helix. These conformational changes would represent the molecular basis for the loss of central neurotoxicity and binding properties to N-type receptors of these mutants (see below).

Probing the role of $sPLA_2$ activity in central neurotoxicity of OS_2

Our first aim was to analyze the role of $sPLA_2$ activity by preparing catalytically inactive mutants of OS_2 and determining whether these mutants are still neurotoxic when injected intracerebroventricularly into mice. OS_2 is one of the most neurotoxic $sPLA_2$s by this route (30). The results obtained with the mutant H48Q indicate a clear dissociation, since this mutant is only 16-fold less neurotoxic, but 500-fold less catalytically active than WT OS_2. This result is in accordance with the partial dissociation obtained in the past by alkylation of His-48 on several neurotoxic $sPLA_2$s (41–43,96). The results obtained with the mutants G30S and D49K support the view that $sPLA_2$ activity may be required for neurotoxicity since these two mutants have lost both properties (Table 2 and Table 3). However, as explained above, their loss of neurotoxicity may be due to a conformational change at the surface of the protein induced by the point mutation. It may be also possible that surface residues from the Ca^{2+}-loop including G30 more directly participate to the interaction with the neurotoxic cellular targets. Our data obtained with the central and C-terminal chimera further indicate that there is no direct correlation between enzymatic activity and neurotoxicity. Indeed, the central chimera is at least as active as WT OS_2, but has a 35-fold lower neurotoxicity, and the C-terminal chimera has 500-fold lower activity, but only 16-fold less central neurotoxicity. The fact that both the H48Q mutant and the C-terminal chimera have dramatically reduced enzymatic activity on both anionic and zwitterionic phospholipids likely indicates that their catalytic activity is also very low on cellular phospholipids *in vivo*, although this hypothesis should deserve attention in future studies.

The fact that the H48Q mutant and the C-terminal chimera retain most of the central neurotoxicity of WT OS_2, yet show a much more drastic loss in enzymatic activity on all types of anionic and zwitterionic phospholipid substrates (Table 3) indicates that the enzymatic activity is at most a minor factor involved in central neurotoxicity. If we assume that the 16-fold decrease in central neurotoxicity is due to mutations that cause a small structural change in the neurotoxic site of OS_2 which in turn affects the energetics of its binding to brain components required for central neurotoxicity (a 16-fold factor would correspond to a loss of binding energy to brain component(s) of about 1.5 kcal/mol), the catalytic activity of OS_2 would in fact not be required for central neurotoxicity. According to this hypothesis, the neurotoxic action of OS_2 would simply be due to the specific binding of the $sPLA_2$ to its cellular neuronal targets via the neurotoxic site. OS_2, and possibly other neurotoxic $sPLA_2$s, would then behave like many other distinct neurotoxins which are devoid of catalytic activity and bind to specific membrane targets (97–99). This view is reminiscent of the situation observed for the K-49 myotoxic $sPLA_2$s, which exert their myotoxic effect independently of enzymatic activity, but via a myotoxic site located on the C-terminal region of the $sPLA_2$ (36). It is also reminiscent of the fact that the major anticoagulant properties of $sPLA_2$s do not require enzymatic activity, but rather a specific binding to factor Xa (15,100). The production of OS_2 mutants fully devoid of catalytic activity yet still highly neurotoxic is however required to validate this hypothesis.

Mapping the region of OS_2 essential for its central neurotoxicity

The results obtained with the chimeras indicate that the N-terminal region of OS_2 contains key residues which are not present in OS_1 and constitute the major structural determinants of the neurotoxic site. The slight decrease in central neurotoxicity observed for the central and C-terminal chimeras suggests that a few residues from these regions contribute to neurotoxicity, or that their mutations mildly affect the structure of the N-terminal region. Similarly, the results obtained with the mutants G30S and D49K indicate that strictly conserved residues from the Ca^{2+}-loop are either key structural elements of the neurotoxic site or that these residues contribute to the conformational stability of the neurotoxic site. The neurotoxic site of OS_2 is thus overlapping with the interfacial binding domain, and this explains why the $sPLA_2$ activity seems to be involved, although no obvious correlation can be found. The proposed location of the OS_2 neurotoxic site agrees with several other studies based on protein sequence alignments, crystal structure analyses, amino acid modifications and site-directed mutagenesis (35,[43],[61],[67],[84],[96],101). However, it is in apparent contradiction with some studies indicating that the β-wing or the C-terminal domains are involved ((47,[102],103) and references therein). Importantly, it should be noted that the modifications or mutations performed in these latter studies led in most cases to a small decrease in neurotoxicity, lower than 10-fold, which is comparable to the relatively modest 16- and 35-fold decreases observed here for the central and C-terminal chimeras. Our results using the single point mutations D49K, G30S and the chimeras led to more consistent decrease or even complete loss of neurotoxicity, unambiguously indicating that the Ca^{2+}-loop and the N-terminal region are crucially involved. Finally, it is remarkable that the single point mutation G30S completely inactivates OS_2, and virtually converts this latter into OS_1 in terms of molecular and functional properties (except for the antimalarial activity, see below). The same is also observed for the N-terminal chimera, and a similar result was previously observed for the bee venom $sPLA_2$ when mutated at the functionally homologous glycine-12 (67).

Binding properties of the OS_2 mutants and chimeras to M-type and N-type receptors and relationship with central neurotoxicity

The pancreatic $sPLA_2$ has previously been shown to bind to the M-type receptor through residues located in or close to the Ca^{2+}-loop, and this binding leads to an inhibition of $sPLA_2$ activity (25,32). However, the binding does not appear to be related to enzymatic activity since both catalytically active and inactive enzymes can bind to the M-type receptor (25,[32],50). In the case of OS_2, we also found that the active site mutants can bind to rabbit or mouse M-type receptors with high affinity (Table 4). Since the active site residues, in particular His-48 and Asp-49, probably do not directly interact with residues from the receptor, it is likely that the small shifts in affinity observed for the H48Q and D49K mutants (Table 4) are due to mutation-induced conformational changes occurring on the molecular surface of OS_2 (see above). For chimeras, we found that they all bind to the M-type receptor. This result was expected since both OS_1 and OS_2 bind to this receptor (58). Since OS_1 and both neurotoxic and non neurotoxic mutants of OS_2 can bind with similar affinities to this receptor while several other neurotoxic $sPLA_2$s do not bind to this receptor (58), it is unlikely that the M-type receptor plays a critical role in central neurotoxicity. This contrasts with the proposed role of the M-type receptor in the neurotoxicity of ammodytoxins (39).

The N-type receptors for OS_2 and other neurotoxic $sPLA_2$s were initially identified in rat brain membranes (30), and then in several peripheral tissues and cells (25), including the HeLa P4 cells used for HIV studies (see below). Although not yet cloned, these receptors are likely to form a family of binding proteins with molecular masses distinct from that of the M-type receptor (25). The physiological roles of these receptors are still unclear, but they have been proposed to participate in the neurotoxic and anti-HIV activities of venom $sPLA_2$s (20,30). The binding properties of the different mutants to the N-type receptors expressed in mouse brain microsomal membranes and HeLa P4 cells follow the same trend. As measured by competition studies using iodinated WT OS_2, mutations in the active site decrease the affinity of OS_2 for the N-type receptors, but there is no obvious relationship between receptor affinity and enzymatic activity or interfacial binding to purified lipid vesicles. As discussed above, the shift in affinity may be explained by the fact that mutations in the active site lead to conformational changes in the Ca^{2+}-loop and N-terminal surface of OS_2 that directly affect the binding properties. This explanation appears more likely than the one assuming that the receptor has a molecular arm penetrating directly into the active site. A similar shift in affinity was observed when OS_2 was alkylated by p-bromo-phenacyl-bromide (unpublished data). The absence of relationship between binding and enzymatic activity was also observed for the chimeras, where all three possibilities were found, *ie* loss of both receptor binding and enzymatic activity (N-terminal chimera), loss of binding but not $sPLA_2$ activity (central chimera), or loss of more enzymatic activity than binding (C-terminal chimera).

The apparent discrepancies observed between cross-linking experiments and competition assays with the central and C-terminal chimeras against labeled WT OS_2 prompted us to investigate their direct binding properties. The radiolabeled C-terminal chimera appeared to bind to a minor subset of OS_2 binding sites which displays high affinity for WT OS_2, as well as for the central and C-terminal chimeras, but not for the N-terminal chimera (Fig. 6). These results restore at least partially the correlation between the neurotoxicity and the affinity of OS_2 mutants for this subset of N-type binding sites, and thus suggest that the high affinity binding sites for the C-terminal chimera are more directly involved in central neurotoxicity. The molecular nature and complexity of these binding sites are unknown, but they most likely consist of several proteins, as revealed by the cross-linking experiments and the curvilinear Scatchard plot analysis (Fig. 5 and Fig. 6). The nature of the other subset of WT OS_2 binding sites on which the C-terminal chimera binds with low affinity is also unknown, but the fact that the C-terminal chimera cannot bind efficiently to phospholipid vesicles raises the possibility that they are lipidic in nature. These latter binding sites would not be important for central neurotoxicity. The N-terminal region of OS_2, but not the central region, appears to be critical for the interaction with the two subsets of N-type OS_2 binding sites. On the other hand, the C-terminal region of OS_2 appears important only for binding to the subset which may be lipidic in nature. The critical role of the N-terminal region in central neurotoxicity of OS_2 and interaction with these subsets of N-type receptors is in agreement with the results previously obtained for bee venom $sPLA_2$ (67) and other neurotoxic $sPLA_2$s including taipoxin, Pa-11, crotoxin (104) and ammodytoxins ((105) and references therein). The relationships between the ammodytoxin binding proteins which have been identified (calmodulin, R25 and 14-3-3 protein isoforms) and these subsets of N-type receptors are still unclear. However, our results are in agreement with some of the structure-function studies on ammodytoxins indicating that residues in the N-terminal and C-terminal regions, but not the β-wing (which is within the OS_1/OS_2 region swapped in the central chimera), are involved in binding to calmodulin and R25 (34,[47],84).

Inhibition of neuromuscular transmission and myotoxic effects induced by OS_2

Similarly to many neurotoxic snake venom $sPLA_2$s which are called β-neurotoxins and include β-Bungarotoxin, crotoxin, textilotoxin, notexin, and taipoxin (69,106), we found that OS_2, but not OS_1, acts pre-synaptically on a chick *biventer cervicis* neuromuscular preparation, indicating that OS_2 is also a β-neurotoxin. At 200 nM, we did not observe any evidence for a post-synaptic effect, which fits with the fact that β-neurotoxins exert post-synaptic effects only at concentrations higher than those required for their neurotoxic effects (107). Except for K31L/R34S, none of the OS_2 mutants had any inhibitory effect at this concentration on the chick *biventer cervicis* preparation, hampering the analysis of the structure-function relationships of OS_2 in this system. The relatively low activity of OS_2 in this model is probably linked to the resistance of the chick preparation to the different β-neurotoxins from Australian elapids (106), although we observed that OS_2 is highly neurotoxic when directly injected in chick brain (unpublished data).

Several group IA and IIA venom $sPLA_2$s which are catalytically active (D49) or inactive (K49) act as potent myotoxins that induce muscle necrosis by disrupting the integrity of the plasma membrane (14). The myotoxicity of group I and II $sPLA_2$s may depend on different structural determinants. The primary mechanism of action of both active and inactive group II myotoxic $sPLA_2$s is independent of $sPLA_2$ activity, and is likely to be due to basic and aromatic residues in the C-terminal region that allow the toxin to bind directly to phospholipids or yet ill-defined

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protein targets on the surface of skeletal muscle cells (14,[35],[36],[95],108). On the other hand, all potent myotoxic group I $sPLA_2$s were found to lack the pancreatic loop and to have a cluster of surface residues including R15, V88, A100, N108, and cationic and hydrophobic residues at positions 83 and 109 (109). Here, we found that OS_2, but not OS_1, exerts myotoxic effects with histological and biochemical features similar to those of group I and II $sPLA_2$ myotoxins. OS_2 has most of the residues associated with myotoxicity in group I $sPLA_2$s, and lacks the pancreatic loop, whereas OS_1 lacks V88 and has the pancreatic loop (Fig 1). Like myotoxic group II $sPLA_2$s, OS_2, but not OS_1, also has basic and aromatic residues in its C-terminal region (Fig. 1), including W117 (Fig. 1 and Fig. 2), which probably plays an important role (110, 111). The role of these residues and $sPLA_2$ activity could not be analyzed in detail since all the OS_2 mutants, except K31L-R34S, appeared to lack myotoxic activity. Since most of the $sPLA_2$ mutants that have lost myotoxicity have also lost enzymatic activity, one might conclude at first sight that $sPLA_2$ activity is critical. However, the relationship is still unclear since the central chimera is not myotoxic while it has a high $sPLA_2$ activity. Since most OS_2 mutants bind to the M-type receptor while they are no longer myotoxic, it is unlikely that this receptor is involved in myotoxicity, at least in the mouse. It is also unlikely because the receptor is expressed at very low levels in adult mouse skeletal muscle, and because the very potent K49 myotoxins Ba-II and Ba-IV purified from *Bothrops asper* do not bind to the endogenous or cloned mouse M-type receptor (MR, BL, JMG, and GL, unpublished data).

Overall, the same trend was observed for the different OS2 mutants at the chick neuromuscular junction and for myotoxicity. The fact that all enzymatically inactive OS2 mutants are inefficient suggests that catalytic activity is essential for the two types of toxicity. However, the fact that the central chimera is also inactive in both cases, while it has a high level of enzymatic activity, suggests that both catalytic activity and other cellular factor(s) are critical for peripheral neurotoxicity and myotoxicity, with the central region of OS2 containing some key structural determinants. Importantly, these observations contrast with those observed for central neurotoxicity, where the H48Q mutant and the central and C-terminal chimeras were nearly as active as WT OS2 (see above). This suggests that the neurotoxic mechanisms operating at the peripheral neuromuscular junction and those occuring in the brain may be distinct. Very recently, Montecucco et al. proposed that the enzymatic activity of different venom sPLA2s is a key factor for peripheral neurotoxicity (40). This proposal was based on the fact that equimolar mixtures of lysophospholipids and fatty acids closely mimic the biological effects of venom sPLA2s at the mouse neuromuscular junction. Unfortunately, no mutated neurotoxic sPLA2s were used in this study. This proposal fits our results at the neuromuscular junction, but contrasts with our findings regarding central neurotoxicity. Since Montecucco et al. do not exclude the contribution of sPLA2 receptors in their proposed mechanism of action (40), it may be possible that different receptors are targeted at the peripheral and central nervous systems. The subtype of brain receptors identified with the C-terminal chimera would be one of these receptors.

Anti-malarial and Anti-HIV activity of OS_2 mutants and chimeras

Several venom $sPLA_2$s have been shown to kill the parasite *P. falciparum* during its intra-erythrocytic development (21) by an indirect mechanism mediated by hydrolysis of serum lipoproteins and production of lipid products which are toxic for the parasite (72). Our results with OS_2 and its mutants are in accordance with this mechanism. Indeed, the inhibitory potency of the mutants correlates with their relative decrease in enzymatic activity and is dependent on the presence of serum. In this case, OS_1 and the central chimera are as active as, or even more active than WT OS_2. Whether their effects are related to particular enzymatic properties (Table 3) toward serum lipoproteins requires further investigation.

Our previous studies have shown that several venom $sPLA_2$s can also inhibit HIV replication by a mechanism that is still ill-defined, but that may not be associated with catalytic activity, since catalytically inactive K-49 $sPLA_2$s can inhibit HIV while $sPLA_2$ catalytic products and inhibitors do not block infection (20,37). Among all the $sPLA_2$s tested, bee venom $sPLA_2$ appears to be the most potent with an IC_{50} of 0.6 nM. WT OS_2 is about 60-fold less potent with an IC_{50} of 35 nM, yet it is more potent than OS_1 (Table 2). This relatively low inhibitory activity has prevented a detailed analysis of the OS_2 antiviral structure-function relationships. Nevertheless, our results show that, as for central neurotoxicity, the N-terminal, but not the central and C-terminal regions of OS_2 is critical. At first sight, our results support the view that catalytic activity is important. However, the result with the C-terminal chimera which is still potent against HIV while it has a low enzymatic activity supports the view that this mutant binds to a subtype of N-receptors which may be similar to the one identified in mouse brain.

Concluding remarks

Our results demonstrate that some biological effects of OS_2 are due to its enzymatic activity (for example the antimalarial effect), while others including central neurotoxicity are more related to specific binding to target cells than enzymatic activity. The most obvious dissociation between enzymatic activity and central neurotoxicity was observed with the H48Q mutant and the C-terminal chimera. Based on the chimera approach, both the N- and C-terminal regions are important for catalytic activity, but only the N-terminal is critical for central neurotoxicity and anti-HIV activity. Based on G30S and D49K mutants, the Ca^{2+}-loop appears important for all activities, although it is highly conserved among $sPLA_2$s. Based on the central chimera, the β-wing structure did not appear to play a key role in the molecular properties or biological activities of OS_2. Amazingly, no clear role has been so far attributed to this particular structure or to the surface residues located in the two large alpha-helices on the back of the $sPLA_2$ (grey scaffold in Fig. 2). This suggests that these two regions play only a structural role, serving as a rigid scaffold on which the functionally important and flexible domains containing the key residues involved in the specific $sPLA_2$ functions are anchored (92). Finally, our findings may help to develop novel strategies to neutralize the toxicity of venom $sPLA_2$s (28) or to engineer $sPLA_2$s with more specific functions. It will be interesting to analyze in particular i) the effect of $sPLA_2$ competitive inhibitors or soluble M-type receptors on the biological effects of OS_2 and other $sPLA_2$s, and ii) the effects of peptides from the N-terminal alpha helix or the Ca^{2+}-loop for their neurotoxic or anti-HIV properties, or for their inhibition of the $sPLA_2$ action, as these peptides may be agonists or antagonists. Short peptides derived from the C-terminal region of myotoxic K49 $sPLA_2$s where are (36,112).

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The abbreviations used are

ACN	acetonitrile
CD	circular dichroism
DSS	Suberic acid bis-*N*-hydroxysuccinimide ester

HIV-1	human immunodeficiency virus-1
LD_{100}	lethal dose for 100% of the population tested
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
OS_1	*Oxyuranus scutellatus scutellatus* toxin 1
OS_2	*Oxyuranus scutellatus scutellatus* toxin 2
$recOS_2$	recombinant OS_2
vOS_2	venom OS_2
POPG/S/C	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol /serine /choline
$sPLA_2$	secreted phospholipase A_2
TFA	trifluoracetic acid
WT	Wild-Type

REFERENCES

1. Valentin E, Lambeau G. Increasing molecular diversity of secreted phospholipases A2 and their receptors and binding proteins. Biochim. Biophys. Acta 2000;1488:59–70. [PubMed: 11080677]
2. Kini RM. Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes. Toxicon 2003;42:827–840. [PubMed: 15019485]
3. Six DA, Dennis EA. The expanding superfamily of phospholipase A2 enzymes: classification and characterization. Biochim. Biophys. Acta 2000;1488:1–19. [PubMed: 11080672]
4. Zadori Z, Szelei J, Lacoste MC, Li Y, Gariepy S, Raymond P, Allaire M, Nabi IR, Tijssen P. A viral phospholipase A2 is required for parvovirus infectivity. Dev. Cell 2001;1:291–302. [PubMed: 11702787]
5. Soragni E, Bolchi A, Balestrini R, Gambaretto C, Percudani R, Bonfante P, Ottonello S. A nutrient-regulated, dual localization phospholipase A(2) in the symbiotic fungus Tuber borchii. Embo J 2001;20:5079–5090. [PubMed: 11566873]
6. Nagiec MJ, Lei B, Parker SK, Vasil ML, Matsumoto M, Ireland RM, Beres SB, Hoe NP, Musser JM. Analysis of a novel prophage-encoded group A Streptococcus extracellular phospholipase A(2). J. Biol. Chem 2004;279:45909–45918. [PubMed: 15304506]
7. Hanasaki K, Arita H. Biological functions of group X secretory PLA2. Adv. Exp. Med. Biol 2003;525:93–96. [PubMed: 12751743]
8. Rouault M, Bollinger JG, Lazdunski M, Gelb MH, Lambeau G. Novel mammalian group XII secreted phospholipase A2 lacking enzymatic activity. Biochemistry 2003;42:11494–11503. [PubMed: 14516201]
9. Diaz BL, Arm JP. Phospholipase A2. Prostaglandins Leukot. Essent. Fatty Acids 2003;69:87–97. [PubMed: 12895591]
10. Kudo I, Murakami M. Phospholipase A2 enzymes. Prostaglandins Other Lipid Mediat 2002;68–69:3–58.
11. Singer AG, Ghomashchi F, Le Calvez C, Bollinger J, Bezzine S, Rouault M, Sadilek M, Nguyen E, Lazdunski M, Lambeau G, Gelb MH. Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2. J. Biol. Chem 2002;277:48535–48549. [PubMed: 12359733]
12. Murakami M, Masuda S, Shimbara S, Ishikawa Y, Ishii T, Kudo I. Cellular distribution, post-translational modification, and tumorigenic potential of human group III secreted phospholipase A (2). J. Biol. Chem 2005;280:24987–24998. [PubMed: 15863501]
13. Chwetzoff S, Tsunasawa S, Sakiyama F, Menez A. Nigexine, a phospholipase A2 from cobra venom with cytotoxic properties not related to esterase activity. Purification, amino acid sequence, and biological properties. J. Biol. Chem 1989;264:13289–13297. [PubMed: 2753914]

14. Gutierrez JM, Ownby CL. Skeletal muscle degeneration induced by venom phospholipases A2: insights into the mechanisms of local and systemic myotoxicity. Toxicon 2003;42:915–931. [PubMed: 15019491]
15. Kini RM. Structure-function relationships and mechanism of anticoagulant phospholipase A2 enzymes from snake venoms. Toxicon 2005;45:1147–1161. [PubMed: 15922780]
16. Zuliani JP, Fernandes CM, Zamuner SR, Gutierrez JM, Teixeira CF. Inflammatory events induced by Lys-49 and Asp-49 phospholipases A2 isolated from Bothrops asper snake venom: role of catalytic activity. Toxicon 2005;45:335–346. [PubMed: 15683872]
17. Rizzo MT, Nguyen E, Aldo-Benson M, Lambeau G. Secreted phospholipase A2 induces vascular endothelial cell migration. Blood 2000;96:3809–3815. [PubMed: 11090064]
18. Rufini S, Cesaroni MP, Balestro N, Luly P. Proliferative effect of ammodytin L from the venom of *Vipera ammodytes* on 208F rat fibroblasts in culture. Biochem. J 1996;320:467–472. [PubMed: 8973554]
19. Santamaria C, Larios S, Angulo Y, Pizarro-Cerda J, Gorvel JP, Moreno E, Lomonte B. Antimicrobial activity of myotoxic phospholipases A2 from crotalid snake venoms and synthetic peptide variants derived from their C-terminal region. Toxicon 2005;45:807–815. [PubMed: 15904676]
20. Fenard D, Lambeau G, Valentin E, Lefebvre JC, Lazdunski M, Doglio A. Secreted phospholipases A2, a new class of HIV inhibitors that block virus entry into host cells. J. Clin. Invest 1999;104:611–618. [PubMed: 10487775]
21. Deregnaucourt C, Schrevel J. Bee venom phospholipase A2 induces stage-specific growth arrest of the intraerythrocytic Plasmodium falciparum via modifications of human serum components. J. Biol. Chem 2000;275:39973–39980. [PubMed: 10988294]
22. Valentin E, Lambeau G. What can venom phospholipases A2 tell us about the functional diversity of mammalian secreted phospholipases A2? Biochimie 2000;82:815–831. [PubMed: 11086212]
23. Jeng TW, Hendon RA, Fraenkel-Conrat H. Search for relationships among the hemolytic, phospholipolytic, and neurotoxic activities of snake venoms. Proc. Natl. Acad. Sci. U S A 1978;75:600–604. [PubMed: 273221]
24. Kini RM, Evans HJ. A model to explain the pharmacological effects of snake venom phospholipases A2. Toxicon 1989;27:613–635. [PubMed: 2665186]
25. Lambeau G, Lazdunski M. Receptors for a growing family of secreted phospholipases A2. Trends Pharmacol. Sci 1999;20:162–170. [PubMed: 10322502]
26. Stefansson S, Kini RM, Evans HJ. The basic phospholipase A2 from Naja nigricollis venom inhibits the prothrombinase complex by a novel nonenzymatic mechanism. Biochemistry 1990;29:7742–7746. [PubMed: 2271532]
27. Sribar J, Sherman NE, Prijatelj P, Faure G, Gubensek F, Fox JW, Aitken A, Pungercar J, Krizaj I. The neurotoxic phospholipase A2 associates, through a non-phosphorylated binding motif, with 14-3-3 protein gamma and epsilon isoforms. Biochem. Biophys. Res. Commun 2003;302:691–696. [PubMed: 12646224]
28. Lizano S, Domont G, Perales J. Natural phospholipase A(2) myotoxin inhibitor proteins from snakes, mammals and plants. Toxicon 2003;42:963–977. [PubMed: 15019494]
29. Rehm H. Molecular aspects of neuronal voltage-dependent K^+ channels. Eur. J. Biochem 1991;202:701–713. [PubMed: 1765087]
30. Lambeau G, Barhanin J, Schweitz H, Qar J, Lazdunski M. Identification and properties of very high affinity brain membrane-binding sites for a neurotoxic phospholipase from the Taipan venom. J. Biol. Chem 1989;264:11503–11510. [PubMed: 2544597]
31. Cupillard L, Mulherkar R, Gomez N, Kadam S, Valentin E, Lazdunski M, Lambeau G. Both group IB and group IIA secreted phospholipases A2 are natural ligands of the mouse M-type receptor. J. Biol. Chem 1999;274:7043–7051. [PubMed: 10066760]
32. Hanasaki K, Arita H. Phospholipase A2 receptor: a regulator of biological functions of secretory phospholipase A2. Prostaglandins Other Lipid Mediat 2002;68–69:71–82.
33. Soares AM, Giglio JR. Chemical modifications of phospholipases A2 from snake venoms: effects on catalytic and pharmacological properties. Toxicon 2003;42:855–868. [PubMed: 15019487]

34. Prijatelj P, Krizaj I, Kralj B, Gubensek F, Pungercar J. The C-terminal region of ammodytoxins is important but not sufficient for neurotoxicity. Eur. J. Biochem 2002;269:5759–5764. [PubMed: 12444963]
35. Chioato L, Ward RJ. Mapping structural determinants of biological activities in snake venom phospholipases A2 by sequence analysis and site directed mutagenesis. Toxicon 2003;42:869–883. [PubMed: 15019488]
36. Lomonte B, Angulo Y, Calderon L. An overview of lysine-49 phospholipase A2 myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. Toxicon 2003;42:885–901. [PubMed: 15019489]
37. Fenard D, Lambeau G, Maurin T, Lefebvre JC, Doglio A. A peptide derived from bee venom-secreted phospholipase A2 inhibits replication of t-cell tropic hiv-1 strains via interaction with the CXCR4 chemokine receptor. Mol. Pharmacol 2001;60:341–347. [PubMed: 11455021]
38. Petan T, Krizaj I, Gelb MH, Pungercar J. Ammodytoxins, potent presynaptic neurotoxins, are also highly efficient phospholipase A2 enzymes. Biochemistry 2005;44:12535–12545. [PubMed: 16156665]
39. Krizaj I, Gubensek F. Neuronal receptors for phospholipases A(2)and beta-neurotoxicity. Biochimie 2000;82:807–814. [PubMed: 11086211]
40. Rigoni M, Caccin P, Gschmeissner S, Koster G, Postle AD, Rossetto O, Schiavo G, Montecucco C. Equivalent effects of snake PLA2 neurotoxins and lysophospholipid-fatty acid mixtures. Science 2005;310:1678–1680. [PubMed: 16339444]
41. Fohlman J, Eaker D, Dowdall MJ, Lullmann-Rauch R, Sjodin T, Leander S. Chemical modification of taipoxin and the consequences for phospholipase activity, pathophysiology, and inhibition of high-affinity choline uptake. Eur. J. Biochem 1979;94:531–540. [PubMed: 428399]
42. Chang CC, Su MJ. Presynaptic toxicity of the histidine-modified, phospholipase A2-inactive, beta-bungarotoxin, crotoxin and notexin. Toxicon 1982;20:895–905. [PubMed: 6129723]
43. Rosenberg P, Ghassemi A, Condrea E, Dhillon D, Yang CC. Do chemical modifications dissociate between the enzymatic and pharmacological activities of beta bungarotoxin and notexin? Toxicon 1989;27:137–159. [PubMed: 2718187]
44. Renetseder R, Dijkstra BW, Huizinga K, Kalk KH, Drenth J. Crystal structure of bovine pancreatic phospholipase A2 covalently inhibited by p-bromo-phenacyl-bromide. J. Mol. Biol 1988;200:181–188. [PubMed: 3379639]
45. Zhao H, Tang L, Wang X, Zhou Y, Lin Z. Structure of a snake venom phospholipase A2 modified by p-bromo- phenacyl-bromide [In Process Citation]. Toxicon 1998;36:875–886. [PubMed: 9663694]
46. Tatulian SA. Structural effects of covalent inhibition of phospholipase A2 suggest allosteric coupling between membrane binding and catalytic sites. Biophys. J 2003;84:1773–1783. [PubMed: 12609879]
47. Ivanovski G, Petan T, Krizaj I, Gelb MH, Gubensek F, Pungercar J. Basic amino acid residues in the beta-structure region contribute, but not critically, to presynaptic neurotoxicity of ammodytoxin A. Biochim. Biophys. Acta 2004;1702:217–225. [PubMed: 15488774]
48. Kolko M, Bruhn T, Christensen T, Lazdunski M, Lambeau G, Bazan NG, Diemer NH. Secretory phospholipase A2 potentiates glutamate-induced rat striatal neuronal cell death in vivo. Neurosci. Lett 1999;274:167–170. [PubMed: 10548416]
49. Fossier P, Lambeau G, Lazdunski M, Baux G. Inhibition of ACh release at an *Aplysia* synapse by neurotoxic phospholipases A2: specific receptors and mechanisms of action. J. Physiol 1995;489:29–40. [PubMed: 8583413]
50. Beck S, Lambeau G, Scholz-Pedretti K, Gelb MH, Janssen MJ, Edwards SH, Wilton DC, Pfeilschifter J, Kaszkin M. Potentiation of TNFalpha -induced sPLA2-IIA expression in mesangial cells by an autocrine loop involving secreted phospholipase A2 and PPARalpha activation. J. Biol. Chem 2003;278:29799–29812. [PubMed: 12782627]
51. Berg OG, Gelb MH, Tsai MD, Jain MK. Interfacial enzymology: the secreted phospholipase A2 paradigm. Chem. Rev 2001;101:2613–2654. [PubMed: 11749391]
52. Lambeau G, Ancian P, Nicolas JP, Beiboer S, Moinier D, Verheij H, Lazdunski M. Structural elements of secretory phospholipases A2 involved in the binding to M-type receptors. J. Biol. Chem 1995;270:5534–5540. [PubMed: 7890672]

53. Chen GT, Inouye M. Role of the AGA/AGG codons, the rarest codons in global gene expression in Escherichia coli. Genes Dev 1994;8:2641–2652. [PubMed: 7958922]

54. Moriyama EN, Powell JR. Codon usage bias and tRNA abundance in Drosophila. J. Mol. Evol 1997;45:514–523. [PubMed: 9342399]

55. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 1989;77:51–59. [PubMed: 2744487]

56. Valentin E, Koduri RS, Scimeca J-C, Carle G, Gelb MH, Lazdunski M, Lambeau G. Cloning and recombinant expression of a novel mouse secreted phospholipase A2. J. Biol. Chem 1999;274:19152–19160. [PubMed: 10383420]

57. Lambeau G, Ancian P, Barhanin J, Lazdunski M. Cloning and expression of a membrane receptor for secretory phospholipases A2. J. Biol. Chem 1994;269:1575–1578. [PubMed: 8294398]

58. Lambeau G, Schmid-Alliana A, Lazdunski M, Barhanin J. Identification and purification of a very high affinity binding protein for toxic phospholipases A2 in skeletal muscle. J. Biol. Chem 1990;265:9526–9532. [PubMed: 2160984]

59. Lind P, Eaker D. complete amino-acid sequence of a non-neurotoxic, non-enzymatic phospholipase A2 homolog from the venom of the Australian tiger snake *Notechis scutatus scutatus*. Eur. J. Biochem 1980;111:403–409. [PubMed: 7007039]

60. Scott DL, Achari A, Vidal JC, Sigler PB. Crystallographic and biochemical studies of the (inactive) Lys-49 phospholipase A2 from the venom of Agkistridon piscivorus piscivorus. J. Biol. Chem 1992;267:22645–22657. [PubMed: 1429612]

61. Georgieva DN, Perbandt M, Rypniewski W, Hristov K, Genov N, Betzel C. The X-ray structure of a snake venom Gln48 phospholipase A2 at 1.9A resolution reveals anion-binding sites. Biochem. Biophys. Res. Commun 2004;316:33–38. [PubMed: 15003507]

62. Janssen MJ, van de Wiel WA, Beiboer SH, van Kampen MD, Verheij HM, Slotboom AJ, Egmond MR. Catalytic role of the active site histidine of porcine pancreatic phospholipase A2 probed by the variants H48Q, H48N and H48K. Protein Eng 1999;12:497–503. [PubMed: 10388847]

63. Edwards SH, Thompson D, Baker SF, Wood SP, Wilton DC. The crystal structure of the H48Q active site mutant of human group IIA secreted phospholipase A2 at 1.5 A resolution provides an insight into the catalytic mechanism. Biochemistry 2002;41:15468–15476. [PubMed: 12501175]

64. Van Den Bergh CJ, Slotboom AJ, Verheij HM, De Haas GH. The role of aspartic acid-49 in the active site of phospholipase A2. A site-specific mutagenesis study of porcine pancreatic phospholipase A2 and the rationale of the enzymatic activity of [lysine49]phospholipase A2 from *Agkistrodon piscivorus piscivorus* venom. Eur. J. Biochem 1988;176:353–357. [PubMed: 3046944]

65. Bekkers AC, Franken PA, Toxopeus E, Verheij HM, De Haas G. The importance of glycine-30 for enzymatic activity of phospholipase A2. Biochim. Biophys. Acta 1991;1076:374–378. [PubMed: 2001385]

66. Van Den Bergh CJ, Slotboom AJ, Verheij HM, De Haas GH. The role of Asp-49 and other conserved amino acids in phospholipases A2 and their importance for enzymatic activity. J. Cell. Biochem 1989;39:379–390. [PubMed: 2722967]

67. Nicolas JP, Lin Y, Lambeau G, Ghomashchi F, Lazdunski M, Gelb MH. Localization of structural elements of bee venom phospholipase A2 involved in N-type receptor binding and neurotoxicity. J. Biol. Chem 1997;272:7173–7180. [PubMed: 9054413]

68. Chizzonite R, Truitt T, Podlaski FJ, Wolitzky AG, Quinn PM, Nunes P, Stern AS, Gately MK. IL-12: monoclonal antibodies specific for the 40-kDa subunit block receptor binding and biologic activity on activated human lymphoblasts. J. Immunol 1991;147:1548–1556. [PubMed: 1715362]

69. Hawgood, B.; Bon, C. Snake Venom Presynaptic Toxins. In: Tu, AT., editor. Handbook of Natural Toxins. New York: Marcel Dekker; 1991. p. 3-52.

70. Gandolfo G, Lambeau G, Lazdunski M, Gottesmann C. Effects on behavior and EEG of single chain phospholipases A2 from snake and bee venoms injected into rat brain. Research for a functional antagonism. Pharmacol. and Toxicol 1996;78:341–347.

71. Gutierrez JM, Lomonte B. phospholipase A2 myotoxins from *Bothrops* snake venoms. Toxicon 1995;33:1405–1424. [PubMed: 8744981]

72. Guillaume C, Deregnaucourt C, Clavey V, Schrevel J. Anti-Plasmodium properties of group IA, IB, IIA and III secreted phospholipases A2 are serum-dependent. Toxicon 2004;43:311–318. [PubMed: 15033330]

73. Viljoen CC, Visser L, Botes DP. An essential tryptophan in the active site of phospholipase A2 from the venom of Bitis gabonica. Biochim. Biophys. Acta 1976;438:424–436. [PubMed: 952941]

74. Nishida S, Tamiya N. Tryptophan residues of phospholipase A2 from the venom of an Australian elapid snake (Pseudechis australis). Toxicon 1991;29:429–439. [PubMed: 1862519]

75. Chang LS, Kuo KW, Chang CC. Identification of functional involvement of tryptophan residues in phospholipase A2 from Naja naja atra (Taiwan cobra) snake venom. Biochim. Biophys. Acta 1993;1202:216–220. [PubMed: 8399382]

76. Liu X, Zhu H, Huang B, Rogers J, Yu BZ, Kumar A, Jain MK, Sundaralingam M, Tsai MD. Phospholipase A2 engineering. Probing the structural and functional roles of N-terminal residues with site-directed mutagenesis, X-ray, and NMR. Biochemistry 1995;34:7322–7334. [PubMed: 7779775]

77. Han SK, Kim KP, Koduri R, Bittova L, Munoz NM, Leff AR, Wilton DC, Gelb MH, Cho W. Roles of Trp31 in high membrane binding and proinflammatory activity of human group V phospholipase A2. J. Biol. Chem 1999;274:11881–11888. [PubMed: 10207008]

78. Janssen MJ, Burghout PJ, Verheij HM, Slotboom AJ, Egmond MR. Introduction of a C-terminal aromatic sequence from snake venom phospholipases A2 into the porcine pancreatic isozyme dramatically changes the interfacial kinetics. Eur. J. Biochem 1999;263:782–788. [PubMed: 10469142]

79. Bezzine S, Bollinger JG, Singer AG, Veatch SL, Keller SL, Gelb MH. On the binding preference of human groups IIA and X phospholipases A2 for membranes with anionic phospholipids. J. Biol. Chem 2002;277:48523–48534. [PubMed: 12244093]

80. Beers SA, Buckland AG, Giles N, Gelb MH, Wilton DC. Effect of tryptophan insertions on the properties of the human group IIA phospholipase A2: mutagenesis produces an enzyme with characteristics similar to those of the human group V phospholipase A2. Biochemistry 2003;42:7326–7338. [PubMed: 12809488]

81. Bollinger JG, Diraviyam K, Ghomashchi F, Murray D, Gelb MH. Interfacial binding of bee venom secreted phospholipase A2 to membranes occurs predominantly by a nonelectrostatic mechanism. Biochemistry 2004;43:13293–13304. [PubMed: 15491136]

82. Snitko Y, Han SK, Lee BI, Cho W. Differential interfacial and substrate binding modes of mammalian pancreatic phospholipases A2: a comparison among human, bovine, and porcine enzymes. Biochemistry 1999;38:7803–7810. [PubMed: 10387020]

83. Canaan S, Nielsen R, Ghomashchi F, Robinson BH, Gelb MH. Unusual mode of binding of human group IIA secreted phospholipase A2 to anionic interfaces as studied by continuous wave and time domain electron paramagnetic resonance spectroscopy. J. Biol. Chem 2002;277:30984–30990. [PubMed: 12039961]

84. Petan T, Krizaj I, Gubensek F, Pungercar J. Phenylalanine-24 in the N-terminal region of ammodytoxins is important for both enzymic activity and presynaptic toxicity. Biochem. J 2002;363:353–358. [PubMed: 11931665]

85. Sa JM, Chioato L, Ferreira TL, De Oliveira AH, Ruller R, Rosa JC, Greene LJ, Ward RJ. Topology of the substrate-binding site of a Lys49-phospholipase A2 influences Ca2+-independent membrane-damaging activity. Biochem. J 2004;382:191–198. [PubMed: 15147240]

86. Qin S, Pande AH, Nemec KN, He X, Tatulian SA. Evidence for the regulatory role of the N-terminal helix of secretory phospholipase A2 from studies on native and chimeric proteins. J. Biol. Chem 2005;280:36773–36783. [PubMed: 16103116]

87. Kuipers OP, Thunnissen MM, de Geus P, Dijkstra BW, Drenth J, Verheij HM, de Haas GH. Enhanced activity and altered specificity of phospholipase A2 by deletion of a surface loop. Science 1989;244:82–85. [PubMed: 2704992]

88. Li Y, Yu BZ, Zhu H, Jain MK, Tsai MD. Phospholipase A2 engineering. Structural and functional roles of the highly conserved active site residue aspartate-49. Biochemistry 1994;33:14714–14722. [PubMed: 7993900]

89. Ward RJ, Chioato L, de Oliveira AH, Ruller R, Sa JM. Active-site mutagenesis of a Lys49-phospholipase A2: biological and membrane-disrupting activities in the absence of catalysis. Biochem. J 2002;362:89–96. [PubMed: 11829743]

90. Huang B, Yu BZ, Rogers J, Byeon IJ, Sekar K, Chen X, Sundaralingam M, Tsai MD, Jain MK. Phospholipase A2 engineering. Deletion of the C-terminus segment changes substrate specificity and uncouples calcium and substrate binding at the zwitterionic interface. Biochemistry 1996;35:12164–12174. [PubMed: 8810924]

91. Yu BZ, Rogers J, Nicol GR, Theopold KH, Seshadri K, Vishweshwara S, Jain MK. Catalytic significance of the specificity of divalent cations as KS* and kcat* cofactors for secreted phospholipase A2. Biochemistry 1998;37:12576–12587. [PubMed: 9730830]

92. Yuan C, Byeon IJ, Poi MJ, Tsai MD. Structural analysis of phospholipase A2 from functional perspective. 2. Characterization of a molten globule-like state induced by site-specific mutagenesis. Biochemistry 1999;38:2919–2929. [PubMed: 10074344]

93. Sekar K, Biswas R, Li Y, Tsai M, Sundaralingam M. Structures of the catalytic site mutants D99A and H48Q and the calcium- loop mutant D49E of phospholipase A2. Acta Crystallogr. D Biol. Crystallogr 1999;55:443–447. [PubMed: 10089353]

94. Chwetzoff S, Couderc J, Frachon P, Menez A. Evidence that the anti-coagulant and lethal properties of a basic phospholipase A2 from snake venom are unrelated. FEBS Lett 1989;248:1–4. [PubMed: 2721666]

95. Ambrosio AL, Nonato MC, de Araujo HS, Arni R, Ward RJ, Ownby CL, de Souza DH, Garratt RC. A molecular mechanism for Lys49-phospholipase A2 activity based on ligand-induced conformational change. J. Biol. Chem 2005;280:7326–7335. [PubMed: 15596433]

96. Soares AM, Mancin AC, Cecchini AL, Arantes EC, Franca SC, Gutierrez JM, Giglio JR. Effects of chemical modifications of crotoxin B, the phospholipase A(2) subunit of crotoxin from Crotalus durissus terrificus snake venom, on its enzymatic and pharmacological activities. Int. J. Biochem. Cell. Biol 2001;33:877–888. [PubMed: 11461830]

97. Massensini AR, Romano-Silva MA, Gomez MV. Sodium channel toxins and neurotransmitter release. Neurochem. Res 2003;28:1607–1611. [PubMed: 14570407]

98. Terlau H, Olivera BM. Conus venoms: a rich source of novel ion channel-targeted peptides. Physiol. Rev 2004;84:41–68. [PubMed: 14715910]

99. Rajendra W, Armugam A, Jeyaseelan K. Neuroprotection and peptide toxins. Brain Res. Brain Res. Rev 2004;45:125–141. [PubMed: 15145623]

100. Mounier CM, Bon C, Kini RM. Anticoagulant venom and mammalian secreted phospholipases A (2): protein- versus phospholipid-dependent mechanism of action. Haemostasis 2001;31:279–287. [PubMed: 11910196]

101. Yang CC, Chang LS. The N-terminal amino group essential for the biological activity of notexin from *Notechis scutatus scutatus* venom. Biochim. Biophys. Acta 1990;1040:35–42. [PubMed: 2378900]

102. Mollier P, Chwetzoff S, Bouet F, Harvey AL, Menez A. Tryptophan 110, a residue involved in the toxic activity but not in the enzymatic activity of notexin. Eur. J. Biochem 1989;185:263–270. [PubMed: 2583182]

103. Yang CC, Chang LS. Dissociation of lethal toxicity and enzymic activity of notexin from *Notechis scutatus scutatus* (Australian tiger snake) venom by modification of tyrosine residues. Biochem. J 1991;280:739–744. [PubMed: 1764038]

104. Tzeng MC, Yen CH, Tsai MD. Binding proteins on synaptic membranes for certain phospholipases A2 with presynaptic toxicity. Adv. Exp. Med. Biol 1996;391:271–278. [PubMed: 8726066]

105. Sribar J, Copic A, Poljsak-Prijatelj M, Kuret J, Logonder U, Gubensek F, Krizaj I. R25 is an intracellular membrane receptor for a snake venom secretory phospholipase A(2). FEBS Lett 2003;553:309–314. [PubMed: 14572642]

106. Hodgson WC, Wickramaratna JC. In vitro neuromuscular activity of snake venoms. Clin. Exp. Pharmacol. Physiol 2002;29:807–814. [PubMed: 12165047]

107. Rowan EG, Harvey AL, Menez A. Neuromuscular effects of nigexine, a basic phospholipase A2 from Naja nigricollis venom. Toxicon 1991;29:371–374. [PubMed: 2048150]

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108. Yamazaki Y, Matsunaga Y, Nakano Y, Morita T. Identification of vascular endothelial growth factor receptor-binding protein in the venom of eastern cottonmouth. A new role of snake venom myotoxic Lys49-phospholipase A2. J. Biol. Chem 2005;280:29989–29992. [PubMed: 16014630]

109. Alape-Giron A, Persson B, Cederlund E, Flores-Diaz M, Gutierrez JM, Thelestam M, Bergman T, Jornvall H. Elapid venom toxins: multiple recruitments of ancient scaffolds. Eur. J. Biochem 1999;259:225–234. [PubMed: 9914497]

110. Lomonte B, Pizarro-Cerda J, Angulo Y, Gorvel JP, Moreno E. Tyr-->Trp-substituted peptide 115–129 of a Lys49 phospholipase A(2) expresses enhanced membrane-damaging activities and reproduces its in vivo myotoxic effect. Biochim. Biophys. Acta 1999;1461:19–26. [PubMed: 10556485]

111. Chioato L, De Oliveira AH, Ruller R, Sa JM, Ward RJ. Distinct sites for myotoxic and membrane-damaging activities in the C-terminal region of a Lys49-phospholipase A2. Biochem. J 2002;366:971–976. [PubMed: 12079495]

112. Nunez CE, Angulo Y, Lomonte B. Identification of the myotoxic site of the Lys49 phospholipase A(2) from Agkistrodon piscivorus piscivorus snake venom: synthetic C-terminal peptides from Lys49, but not from Asp49 myotoxins, exert membrane-damaging activities. Toxicon 2001;39:1587–1594. [PubMed: 11478967]

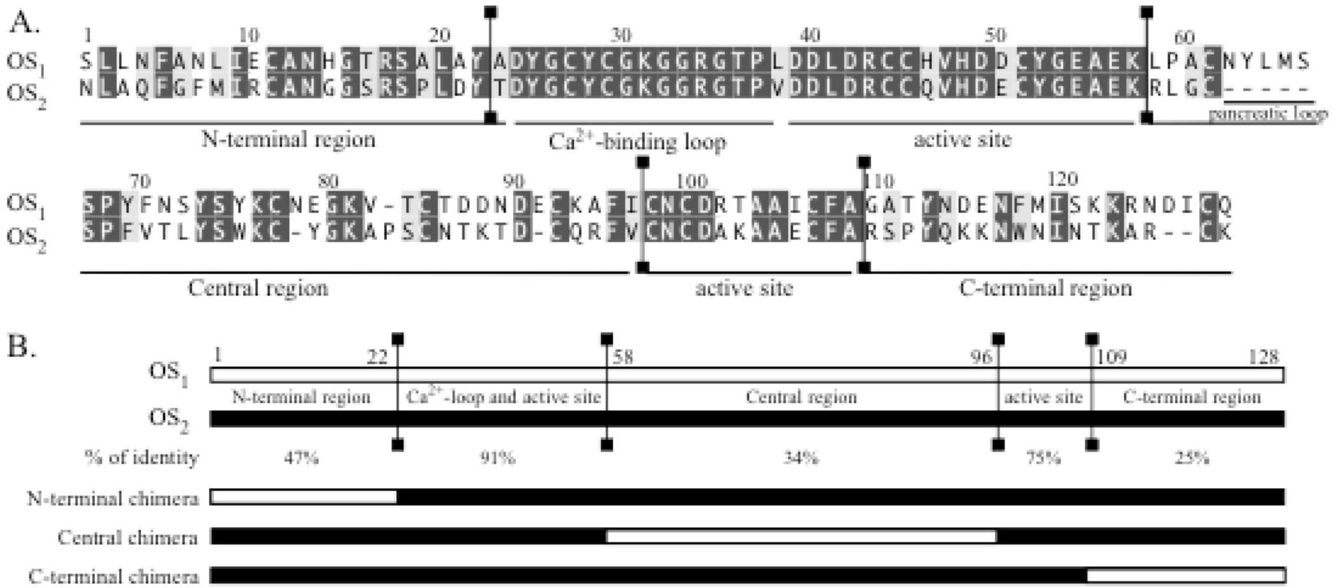

Fig. 1.

Alignment of the amino acid sequences of OS_1 and OS_2 and schematic representation of the chimeras between OS_1 and OS_2. (A) Alignment of the amino acid sequences of OS_1 and OS_2 (52). (B) Schematic representation of the chimeras between OS_1 and OS_2. The conserved and divergent regions between OS_1 and OS_2 are separated by vertical lines in panels A and B. The percentage of identity between the different regions are indicated. The N-terminal (1–22), central (58–96) and C-terminal (109–128) regions of OS_1 have been introduced into the OS_2 sequence to construct N-terminal, central and C-terminal chimeras, respectively.

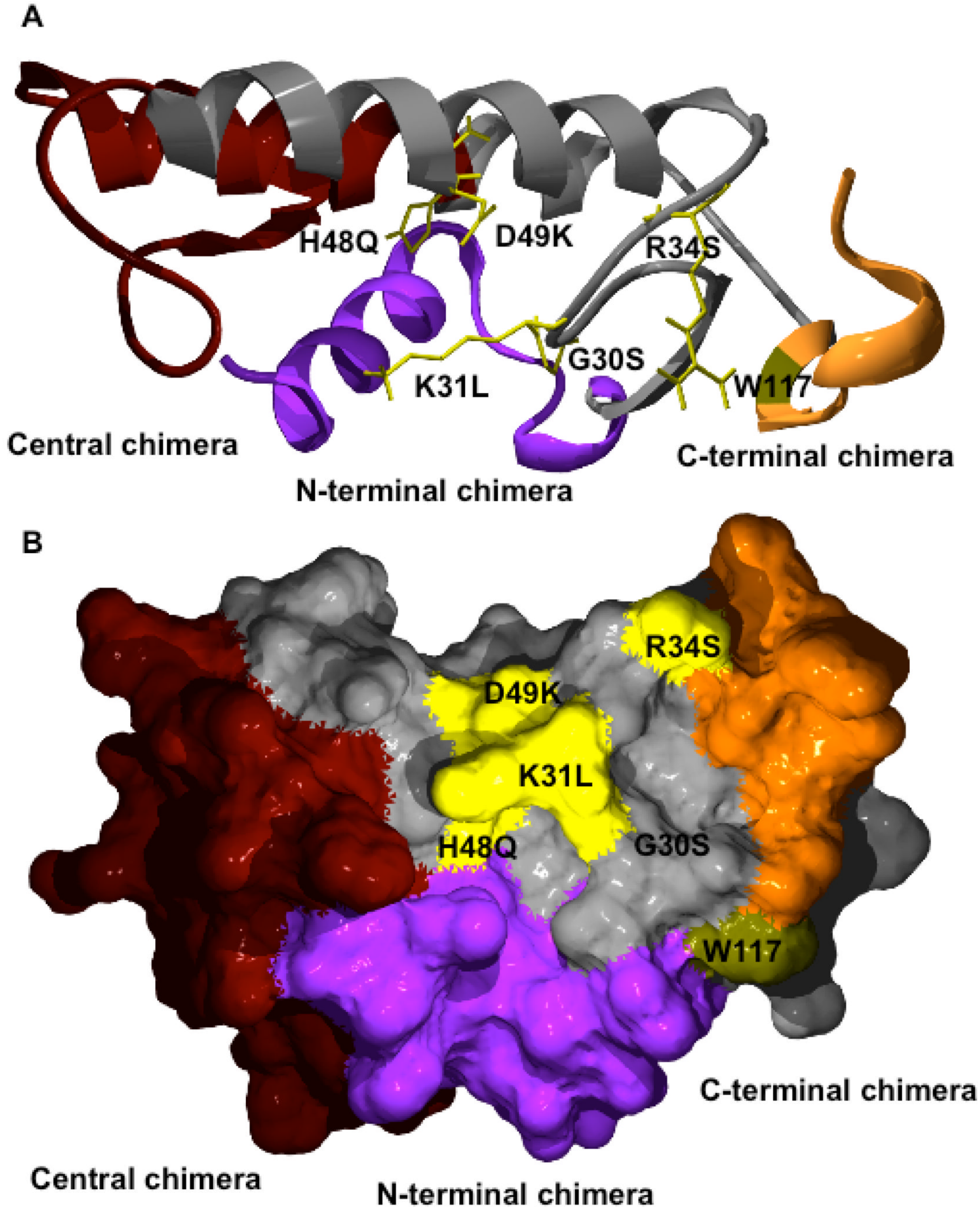

Fig. 2.

Structural model of WT OS_2 showing the location of the different mutations. An OS_2 model was build using the SwissPDB protein viewer software and the crystal structure of notexin (PDB 1AE7) as template. The location of the single point mutations and W117 are shown. The N-terminal, central, and C-terminal regions exchanged in the chimeras are shown in blue, brown, and dark yellow, respectively. The conserved backbone between OS_1 and OS_2 is shown in grey. A, Ribbon view of OS_2 with the putative interfacial binding surface facing down. B, Molecular surface view after rotating the molecule by 90°.

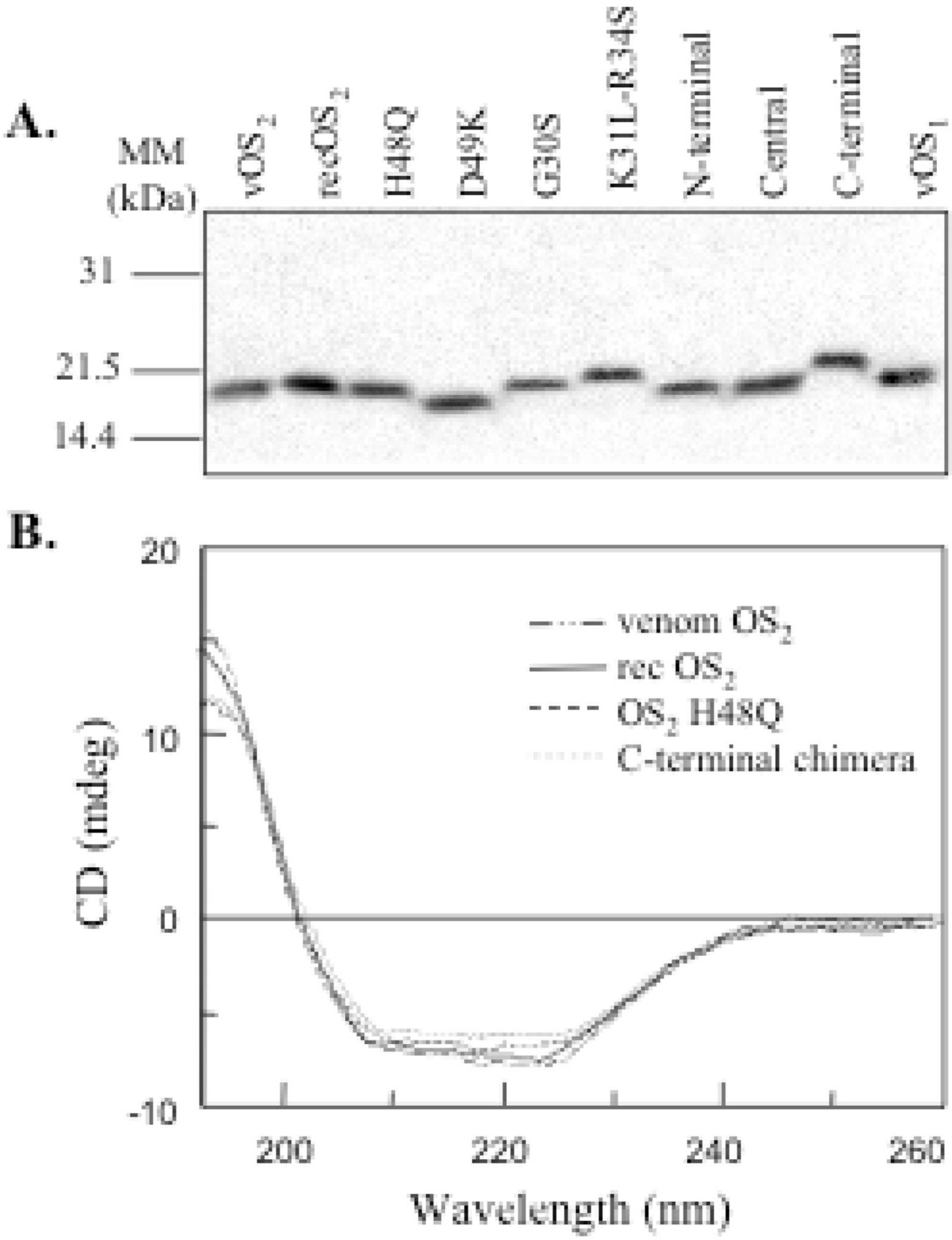

Fig. 3.

Analysis of the OS_2 mutants and chimeras by SDS-PAGE electrophoresis and CD. (A) One μg of $sPLA_2$ was loaded on a 14% SDS-polyacrylamide gel under reducing conditions and stained with Coomassie brilliant blue. The apparent mobility appeared to be very sensitive to the pI of the protein (B) CD spectra of recombinant WT OS_2, H48Q mutant and C-terminal chimera compared to that of venom OS_2. The CD spectra of all the other mutants are identical to those shown here.

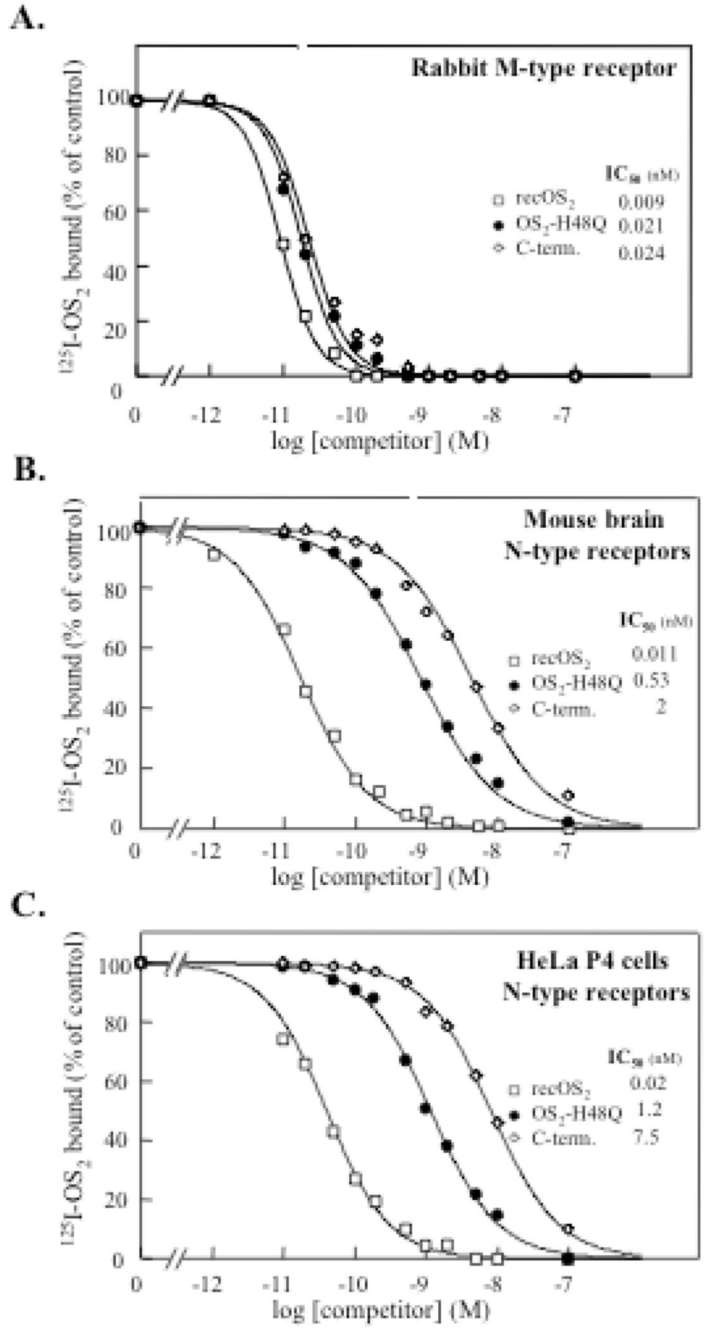

Fig. 4.

Binding properties of OS_2 and its mutants to M- and N-type receptors measured by competition assays with iodinated WT OS_2. Competition experiments between $^{125}I\text{-}OS_2$ and unlabeled WT recombinant OS_2 (*), the mutant H48Q () and the C-terminal chimera (↓) for binding to (A) the cloned rabbit M-type receptor expressed in HEK 293 cells, (B) N-type receptors from mouse brain membranes, and (C) N-type like receptors from HeLa P4 cell membranes. Membranes were incubated with $^{125}I\text{-}OS_2$ and various concentrations of unlabeled competitors. Results are expressed as percentage of the $^{125}I\text{-}OS_2$ specific binding measured in the absence of competitor. The membrane concentration was adjusted to obtain a specific binding of 10–20% of the total radiolabeled ligand added (55 pM). The non specific binding was measured in the presence of 100 nM unlabeled OS_2 and was below 10% of total binding.

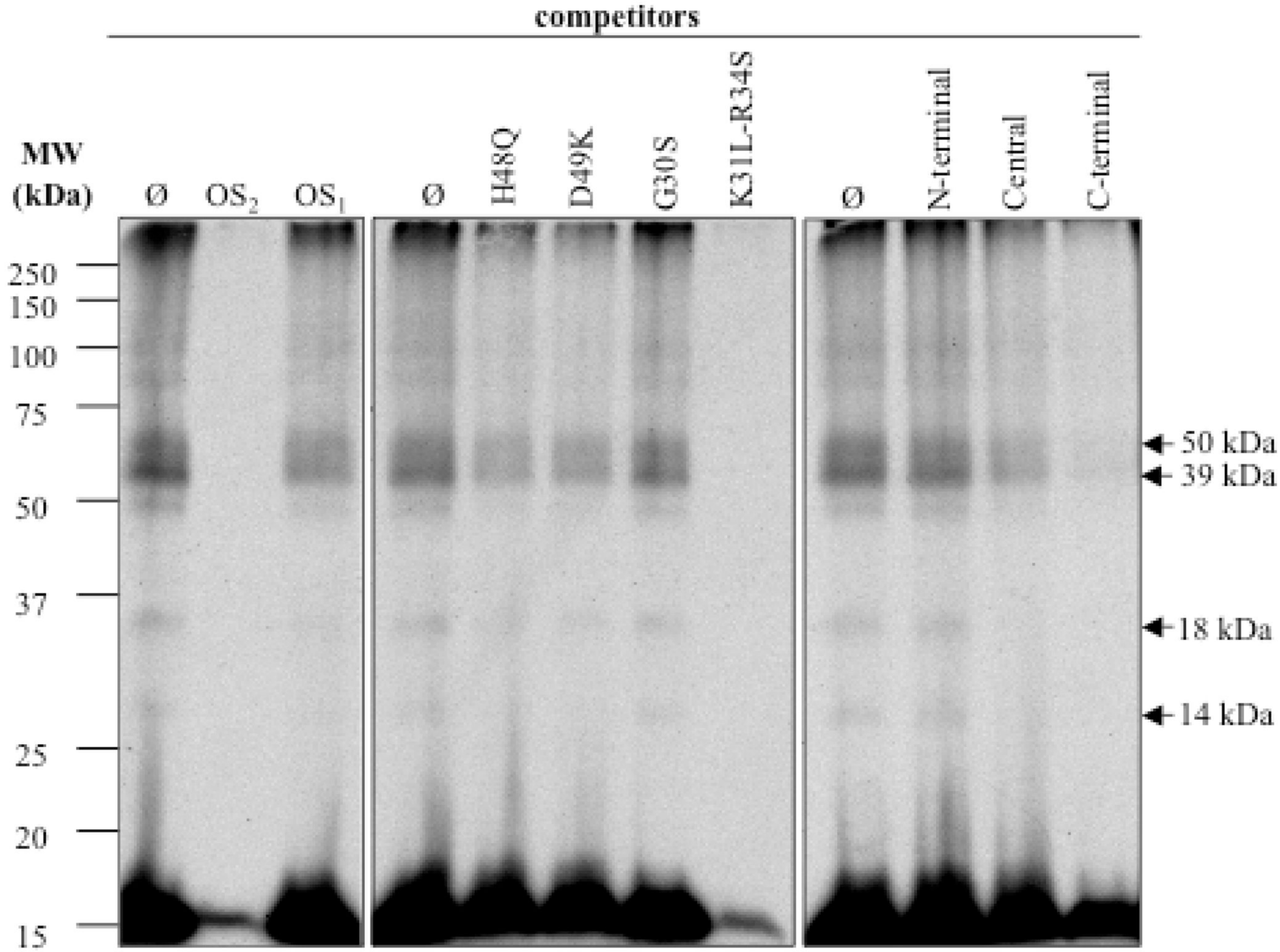

Fig. 5.

Binding properties of WT OS_2 and its mutants as revealed by cross-linking experiments of WT ^{125}I-OS_2 to mouse brain membranes. Membranes (300 µg of protein/ml) were incubated with 200 pM ^{125}I-OS_2 in the absence (Ø) or presence of unlabeled $sPLA_2s$ (300 nM), centrifuged, cross-linked with 200 µM DSS and loaded on a 10% SDS-polyacrylamide gel under reducing conditions. The gel was stained with Coomassie brilliant blue, dried and autoradiographed at −70 °C for 7 days. After correction for the molecular mass of labeled OS_2 assuming a 1:1 stoechiometry, OS_2 binding proteins with molecular masses of 14, 18, 39 and 50 kDa are detected. The most representative experiment out of three experiments performed on two different mouse brain membranes is shown.

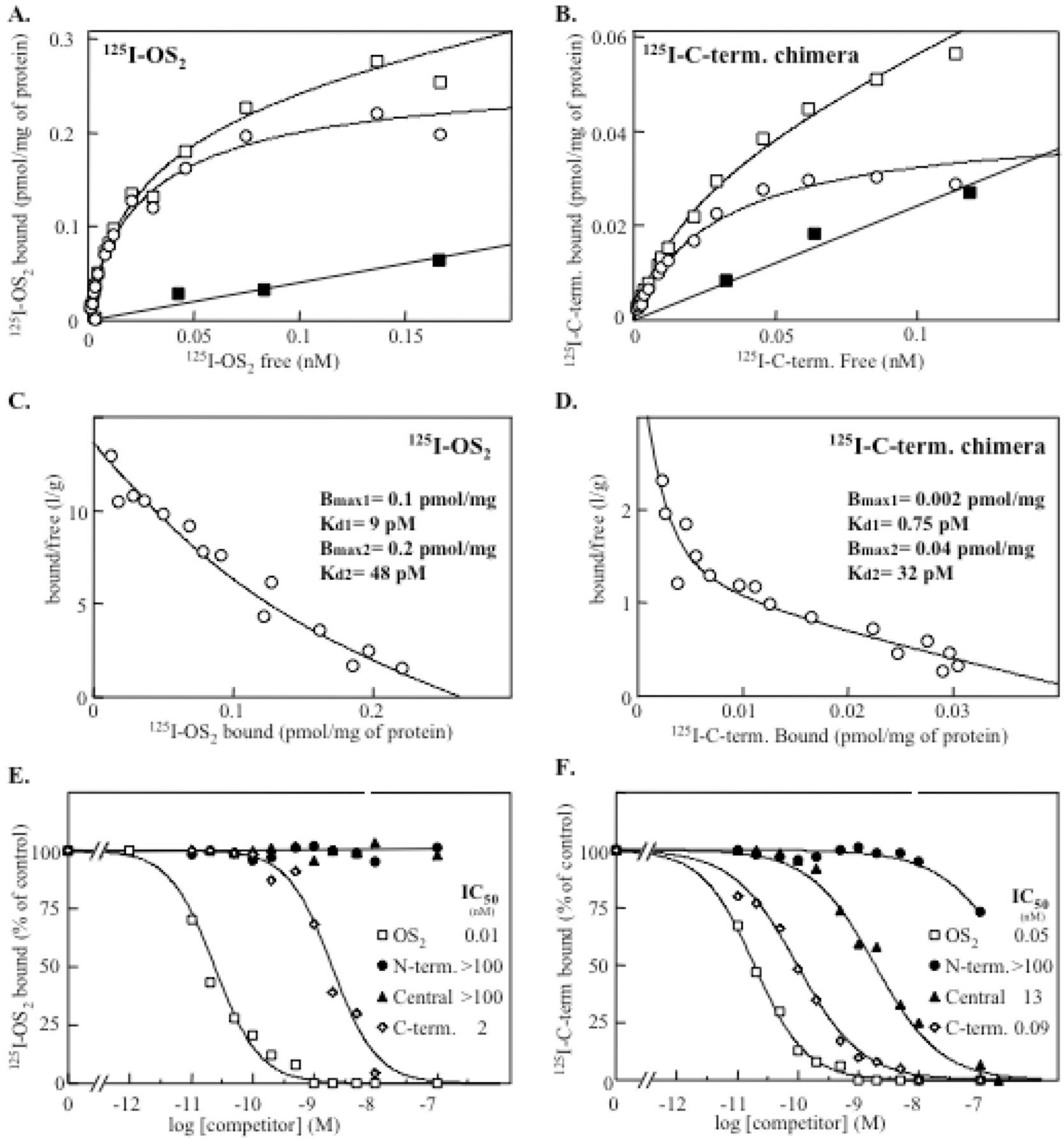

Fig. 6.

Comparison of the binding properties of iodinated WT OS_2 and C-terminal chimera to mouse brain membranes. All of the binding experiments were performed in parallel and on the same membrane preparations (n=3). (A and B), Direct equilibrium binding assays with the two iodinated ligands. Membranes were incubated with various concentrations of labeled ligands in the absence (*) or presence (—) of 100 nM unlabeled homologous ligand. The specific binding (▯) represents the difference between total (*) and non specific binding (—). (C and D), Scatchard plot analysis of the specific binding. (E and F), Competition experiments between iodinated ligands and unlabeled WT recombinant OS_2 and the different chimeras. Membranes were incubated with 50 pM of WT ^{125}I-OS_2 or ^{125}I-C-terminal chimera and various concentrations of unlabeled competitors. Results are expressed as the percentage of ^{125}I-OS_2 specific binding measured in the absence of competitor. The non specific binding was measured in the presence of 100 nM unlabeled homologous ligand and was below 15% of total binding.

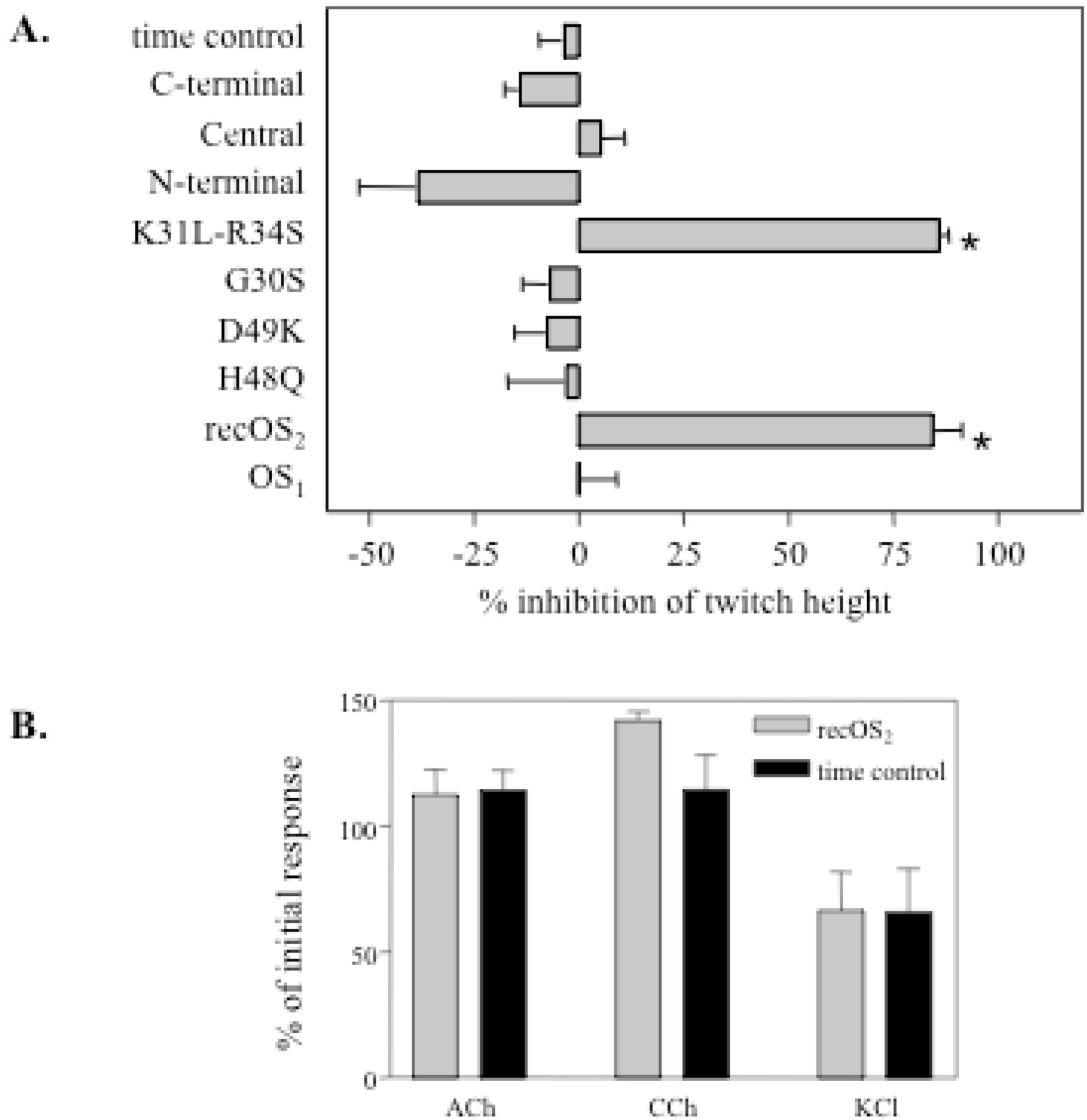

Fig. 7.

Effects of OS_2 and its mutants on neuromuscular transmission in the chick *biventer cervicis* muscle preparation. A, Effects of recombinant OS_2, OS_1, mutants and chimeras (200 nM) on indirect twitches evoked by electrical nerve stimulation. The percentage of inhibition of twitch height was measured 3 h after the addition of $sPLA_2$ to the bath. B, effects of WT recombinant OS_2 on contractures of the chick *biventer cervicis* preparation in response to acetylcholine (ACh, 1 mM), carbachol (CCh, 20mM), and potassium chloride (KCl, 40 mM) compared to time control (—). All experiments were performed in triplicate. * $p< 0.001$ significantly different from time control (unpaired *t*-test assuming unequal variance).

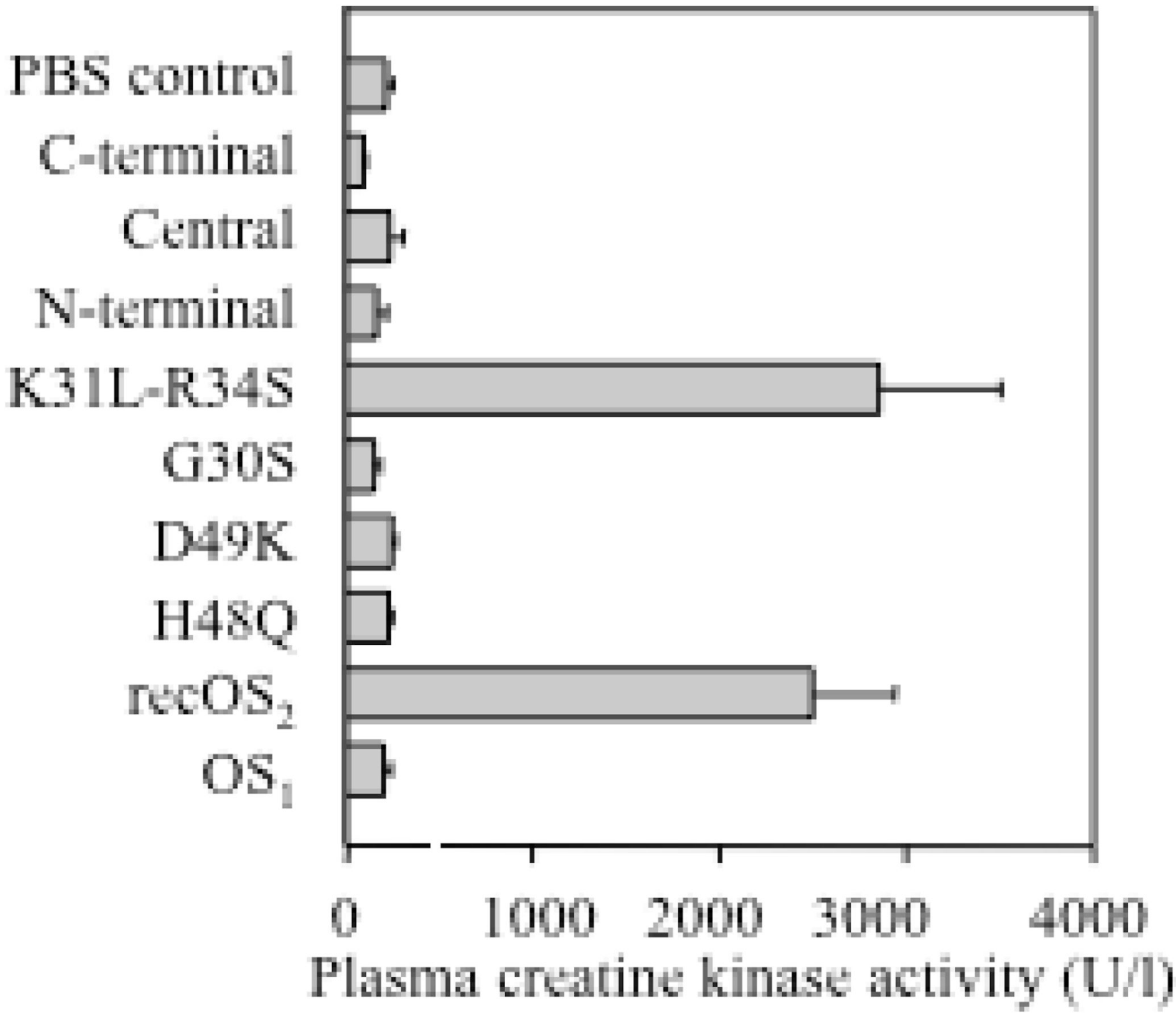

Fig. 8.
Myotoxic effects of OS_2 and its mutants. Three hours after intramuscular injection of $sPLA_2$ (5 µg) into mice, the plasma was collected and creatine kinase activity was measured as an index of myotoxicity. One unit of creatine kinase activity is defined as the amount of enzyme that produces 1 µmol of NADH/min.

TABLE 1

Recombinant production of OS_2 and its mutants

All proteins were produced using the pAB3 E.coli expression system. The yield of production is the amount of pure protein obtained per liter of E. coli culture. The proteins were analyzed by MALDI-TOF mass spectrometry to confirm that all the disulfide bonds are formed and that proteins have not been covalently modified during purification.

$sPLA_2$	Yield of production	Calculated mass	Measured mass	Δmass
	mg/liter	Dalton	Dalton	
OS_2 WT	5	13317.08	13317.86	0.78
OS_2 H48Q	2.6	13308.07	13307.27	0.80
OS_2 D49K	6.4	13330.17	13329.84	0.33
OS_2 G30S	1.3	13347.11	13347.89	0.78
OS_2 K31L-R34S	1.5	13232.96	13233.79	0.83
N-terminal chimera	0.3	13278.02	13277.30	0.72
Central chimera	1	13992.66	13993.29	0.63
C-terminal chimera	2.6	13429.10	13430.12	1.02

TABLE 2
Biological effects of OS_2 and its mutants

Minimal lethal dose (LD_{100}) was measured by intracisternal injection of $sPLA_2$ into adult Balb/C male mice. LD_{100} is defined as the lowest amount of $sPLA_2$ that is lethal to all mice (n≥2), half of this dose being not lethal to mice 4 hours after injection (n≥2). HIV-1 inhibition was determined by infection of HeLa P4 cells with $HIV\text{-}1_{BRU}$ in the absence or presence of various concentrations of $sPLA_2$ for 8 hours. The level of viral infection was evaluated 2 days after infection. Antimalarial activity was measured by incubating various concentrations of $sPLA_2$ with an asynchronous culture of *P. falciparum* (1.5% parasitemia and 2% hematocrit) grown in the presence of 8% human serum or 0.5% AlbuMAX II. The inhibition of growth was determined by ^{3}H-hypoxanthine incorporation. The IC_{50} value is defined as the concentration of $sPLA_2$ that inhibits 50% of $HIV\text{-}1_{BRU}$ infection or parasite growth. The values are representative of at least 2 independent experiments with SEM values lower than 50%.

	LD_{100}	IC_{50} value		
$sPLA_2$s	**Lethality**	**HIV inhibition**	**Antimalarial activity**	
			human serum	**AlbuMAX II**
	μg/kg		nM	
venom OS_2	2	35	3.1	>2,500
rec OS_2 WT	2	33	2.1	>1,250
OS_2 H48Q	33	>1,000	>250	>1,250
OS_2 D49K	>3,330	>1,000	>250	>1,250
OS_2 G30S	3,330	>1,000	>250	>1,250
OS_2 K31L-R34S	7	23	0.1	378
N-terminal	>1,500	>1,000	>250	>1,250
Central	70	110	0.1	680
C-terminal	33	60	17.9	>1,250
venom OS_1	3,500	300	0.07	1,000

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TABLE 3

Enzymatic properties of OS_2 and its mutants

The specific activities were measured on [^{3}H]oleate-radiolabeled *E.coli* membranes and on vesicles composed of the single phospholipid POPG, POPS or POPC using the real-time fluorometric assay employing the fatty acid binding protein. Interfacial binding of $sPLA_2$ was determined by the centrifugation method on sucrose-loaded vesicles of 20 mole% DOetPS/ 80 mole% DOetPC. The results are representative of at least duplicate experiments with SEM values lower than 40%.

$sPLA_2$	**Specific activity**				**Interfacial binding**
	E.coli	**POPG**	**POPS**	**POPC**	***DOetPS/PC***
	dpm/(pmol × min)	μmol/(min×mg)			K_d (mM) [c]
venom OS_2	1090	433	26	53.7	0.023
rec OS_2 WT	1000	471	23	59.1	0.074
OS_2 H48Q	2	1.2	0.05	0.05	0.040
OS_2 D49K	0[a]	nd [b]	nd [b]	nd [b]	no binding at 1 mM
OS_2 G30S	2.3	0.60	0.03	0.03	no binding at 1 mM
OS_2 K31L-R34S	950	169	3.10	61.8	0.007
N-term. chimera	1	2.60	0.003	0.02	no binding at 1 mM
Central chimera	5060	131	5.8	86	0.015
C-term. chimera	1.9	0.01	0.17	0.18	no binding at 1 mM
venom OS_1	10	2.40	2.2	9.7	no binding at 1 mM

[a] No $sPLA_2$ activity is detected at 1 μM after 2 h of incubation.

[b] Not determined.

[c] The indicated K_d values are averaged values of K_d values measured by SDS-PAGE analysis (for $sPLA_2$s with low specific activity), or by both SDS-PAGE and $sPLA_2$ assays (for $sPLA_2$s with high specific activity).

TABLE 4

Binding properties of OS_2 and its mutants to M-type and N-type receptors

Competition binding assays between iodinated OS_2 and unlabeled mutants or chimeras were performed on recombinant M-type receptor or N-type receptors from mouse brain membranes and Hela P4 cell lysates. The $K_{0.5}$ value is defined as the concentration of $sPLA_2$ competitor which inhibits 50% of the specific binding. $K_{0.5}$ values are representative of at least three independent experiments with SEM values lower than 30%.

$sPLA_2$s	$K_{0.5}$ value			
	Rabbit M-type	Mouse M-type	Mouse N-type	HelaP4 N-type
	nM			
Venom OS_2	0.009	0.07	0.011	0.02
rec OS_2 WT	0.009	0.07	0.011	0.02
OS_2 H48Q	0.021	0.19	0.53	1.2
OS_2 D49K	0.026	0.49	13.5	0.96
OS_2 G30S	0.035	0.2	200	400
OS_2 K31L-R34S	0.008	0.047	0.045	0.028
N-terminal chimera	0.19	1.4	>500	>500
Central chimera	0.037	2.2	>300	>300
C-terminal chimera	0.024	0.03	2	7.5
venom OS_1	0.1	0.15	>1000	>1000