Determination of the ability of Gangliosides as expected inhibitors of naja toxin PLA2 (NV-PLA2) prompted human erythrocyte film harm

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Abstract

We determined the ability of mixed gangliosides (16% GD1b, 19% GT1b, 21% GM1, and 40% GD1a) and individual gangliosides GM1 and GD1b to modulate the NV-PLA2 induced human erythrocyte ghost membrane damage. CM-Sephadex purification of crude Naja naja venom yielded eight peaks of which peak VII, a major phospholipase A2 (NV-PLA2) accounted for 22% of the total protein recovered and 8% of the total PLA2 activity recovered. The membrane damage induced by NV-PLA2 was assessed by measuring the decrease in the relative intensity of fluorescence using cis–parinaric acid (PnA) as a monitor molecule. The RBC membranes isolated from healthy human blood showed 72% damage on treatment with NV-PLA2 (2 μg) when compared to untreated membranes. Mixed gangliosides (18 nM) and GM1 (15 nM) offered 81 and 86% protection respectively, whereas GD1b (20 nM) did not show significant protection. Analysis of membrane bound Na+K+ and Ca2+Mg2+ ATPase indicated a 3 fold and 2 folds decrease in their activities on NV-PLA2 treatment when compared to untreated membranes. Mixed gangliosides restored the Na+ K+ ATPase activity by 78%, whereas GM1 and GD1b offered 74 and 52% restoration respectively. The Ca2+Mg2+ ATPase activity was restored by 80 and 81% with mixed gangliosides and GM1 respectively. GD1b showed only 50% restoration. Mixed gangliosides and GM1 exhibited a significant dose dependent inhibition of NV-PLA2 activity when compared to GD1b. The data show that mixed gangliosides and GM1 were effective in modulating NV-PLA2 induced erythrocyte membrane damage than GD1b.

Keywords: Gangliosides, NV-PLA2, PnA, gangliosides, erythrocyte membrane, Na+ K+ ATPase, Ca2+ Mg2+ ATPase.

INTRODUCTION

Venomous snakebite is a serious medical problem worldwide. Annually 45 - 50,000 people die in India alone due to snake envenomation (Chatterjee et al., 2006; Warrell, 2006). The venom of Indian cobra (Naja naja), of Elapidae family induced fatality extends to other tropical and subtropical countries like Sri Lanka, Thailand and Malaysia (Mukherjee and Maity, 2002; Warrell, 1986). N. naja venom acts through systolic heart arrest, necrosis and severe inflammation (Ownby et al., 1993). Snake venom is broadly divided into three categories based on toxicity from envenomation. These categories are (i) hemotoxins, which promote hemorrhaging primary to ex- extensive local swelling and necrosis, (ii) neurotoxins, which disable muscle contraction and paralyze the heart as well as hinder respiration, and (iii) cardiotoxins, which elicit specific toxicity to cardiac and muscle cells, causing irreversible depolarization of cell membranes (Hati et al., 1999). Irrespective of internal consequences, it also causes extensive local tissue damage and inflammation by the direct action of cytotoxic factors (Dong-Zhong et al., 1997). Snake venoms generally contain more than 90% protein and most of them are enzymes, which are proteinases, phospholipases, sphingomyelinases and hyaluronidases. Among these proteins, Phospholipase A2 (PLA2) are abundant (~40%) in almost all snake venoms (Walter et al., 1999; Murari et al., 2005; Kini, 2005).
PLA₂ plays an important role in phospholipids digestion. They catalyze the hydrolysis of Sn-2 fatty acyl chain of phospholipids to yield a fatty acid and a lysophospholipid. The activation of PLA₂ releases free arachidonate from phospholipids. Arachidonate serves as the substrate for two alternate pathways catalyzed by lipooxygenase or cyclooxygenase resulting in prostaglandins and prostacyclins (Balsinde et al., 2002; Dunn and Broady, 2001; Sribar et al., 2003) thus leading to inflammation. Various experimental studies of PLA₂ on lipid bilayers have shown that membrane surface properties, including membrane fluidity, curvature and membrane induced structural chan-ges (Jensen et al., 2003) depend on membrane binding and extent of PLA₂ activation (Tatulian, 2001; Diaz et al., 2001). Utilizing phospholipid mixtures, the activity and specificity of cobra venom PLA₂ is shown to be depend-ent on the presence of other lipids and their organization in the erythrocyte membranes (Florin-Christensen et al., 2001). The Bothrops asper snake venom PLA₂ activity is found to exert its lytic effect on mouse erythrocytes, thereby inducing asymmetry in the membranes, which alters its normal physiological functions (Adamich and Dennis, 1979; Butron et al., 1993).

Gangliosides comprise a family of acidic glycolipids that are characterized by the presence of sialic acid. They contain both hydrophilic and hydrophobic regions, and they bear a strong negative charge (Fishman and Brady, 1976). They are ubiquitous membrane components in the outer leaflet of lipid bilayer conferring structural integrity and are reported to function in cell – cell interaction re-cognition (Yogeeswaran and Hakamori, 1975) as a differentiation marker (Marchase, 1977; Rosenfelder et al., 1979) and in neuromuscular recognition (Obata, 1977). Gangliosides have been established as cell sur-face receptors and several bacterial toxins have been shown to interact with them especially GM1 the specific receptor for cholera toxin (Orlandi and Fishman, 1993). Gangliosides act as an anti-inflammatory agent by inhibit-ing the edema produced by the injection of bee venom phospholipase A₂ in the rat hindpaw (Correa et al., 1991) and also reduces ethanol induced phospholipase A₂ activity in synaptosomal preparations from mice (Hun-gund et al., 1994). Ganglioside GM1 is shown to inhibit arachidonic acid release when stimulated with maltoxin in bovine aortic endothelial cells (Bressler et al., 1994). In this context it was interesting to know whether gangliosides could inhibit the N. naja venom PLA₂ (NV-PLA₂) induced membrane damage using human erythrocyte ghost as model system. In an attempt to investigate the action of gangliosides on NV-PLA₂, we examined the inhibitory effect of gangliosides on NV-PLA₂ induced human erythrocyte ghost membrane damage and inhibition of NV-PLA₂ activity. The results presented in this report indi-cate that, among the gangliosides tested mixed gangliosides and GM1 act as potential inhibitors of NV-PLA₂ induced erythrocyte membrane damage and also inhibit-
ed the NV-PLA₂ activity.

MATERIALS AND METHODS

Cobra (N. naja) venom was obtained from Irula Snake Catchers Society Limited, Chennai, India. The lyophilized powdered venom was dissolved in 0.9% saline (0.1mg/ml) and preserved at 4°C until further use. Ethylene glycol tetra acetic acid (EGTA), Dibromo-ace
tophenone (DBAP), α-Nitroso-β-napthol, Linolenic acid, α-
Phenanthroline, Phosphaticyl choline, mixed gangliosides, ganglio-side GM1 (Bovine brain, approx. 95% lyophilized), Ganglioside GD1b (Bovine brain, approx. 95% lyophilized), cis – Parinacic acid, CM-Sephadex C-25 and Sephadex G-50 were purchased from Sigma Aldrich Co. USA. All the other reagents used were of analy-
tical grade and were purchased from E – Merck, Germany. Gangli-
osides were dissolved in <0.1% Dimethyl Sulfoxide (DMSO) to the desired concentration. Organic solvents were distilled prior to use. Spectrophotometric measurements were done using Shimadzu double beam Spectrophotometer. Spectrofluorimetric measure-
ments were done using Shimadzu Fluorescece Spectrometer.

Preparation of human erythrocyte ghosts

Human blood was collected from healthy male volunteers between 25 – 30 years of age. Erythrocyte ghost, free of hemoglobin and superoxide dismutase, was prepared by the method of Dodge et al. (1963). Blood in acid citrate dextrose in the ratio 5:1 was centri-fuged at 2500 rpm at 4°C for 15 min, the supernatant was discarded and the pellet was washed 3 – 5 times with isotonic phosphate buffer (310 isomill osmolar, pH 7.4), centrifuged at 2500 rpm at 4°C for 20 min. RBC pellet was suspended in hypotonic buffer (201 milli osmolar) and incubated overnight at room temperature for hypo-ionic hemolysis to take place. Contents were centrifuged at 12000 rpm at 4°C for 20 min; the pellet was dislodged gently and washed twice with hypotonic phosphate buffer, centrifuged at 1500 rpm at 4°C for 20 minutes to remove unlysed RBCs. Supernatant was col-lected and centrifuged at 12000 rpm at 4°C for 20 min and washed twice in 0.9% NaCl. Erythrocyte ghost was suspended in 0.9% NaCl in aliquots and stored at -20°C for further use. The protein content of ghost was estimated by Bradford’s method (Bradford, 1976).

Purification of Naja naja venom PLA₂ (NV-PLA₂)

N. naja venom (500 mg) was dissolved in 5 ml of 0.02 M phosphate buffer (pH 7.0) and applied to a CM-Sephadex column (1.5 X 125 cm) which was eluted stepwise, using phosphate buffers of various molalities and pH values as described in Figure 1. The flow rate was adjusted to 24 ml/h and 2 ml fractions were collected by Waters Fraction Collector. Protein elution was monitored at 280 nm using Shimadzu Spectrophotometer. The enzyme was studied by assaying 0.1ml aliquots from the fractions for PLA₂ activity. Individ-
al fractions of the enzyme peak were pooled, desalted, lyophili-
zed and stored at -20°C.

Fraction VII showed PLA₂ activity and is one of the major peaks containing 22% of the venom protein recovered from CM-Sephadex C-25 column and was named NV-PLA₂. 35mg of NV-PLA₂ in 2 ml of 0.1M NaCl was loaded on a Sephadex G-50 column (1.5 x 95 cm). The protein was eluted with 0.1M NaCl with a flow rate of 30 ml/h and 2.5 ml fractions were collected. The protein elution was moni-tored at 280 nm.

Phospholipase A₂ (PLA₂) assay

PLA₂ assay was done according to Bhat and Gowda method (Bhat
Figure 1. CM-Sephadex C-25 column chromatography of *Naja naja* venom

Assessment of erythrocyte membrane damage

Cis–parinaric acid (PnA) was used as a monitor molecule for the assessment of erythrocyte membrane damage (McKenna et al., 1991). Briefly, PnA was dissolved in <0.1% DMSO at a concentration of 10 mM and saturated with N<sub>2</sub> gas. PnA was added to the membranes (200 μg protein) at a final concentration of 20 μM in the presence of NV-PLA<sub>2</sub> (2 μg) and with or without mixed gangliosides (18nM), GM1 (15nM) and GD1b (20nM), incubated in the dark at room temperature for 15 min. The wavelength for the excitation was 324 nm and the intensity of emitted fluorescence (425 nm) was measured using Shimadzu Fluorescence Spectrometer. The relative intensities of fluorescence from the membranes at each incubation time were normalized in a way that the intensity of fluorescence from the membranes at 0 h incubation without inhibitors was 1.0.

Determination of Na<sup>+</sup>K<sup>+</sup>ATPase activity

200 μg of membrane protein were preincubated with or without mixed gangliosides (18 nM), GM1 (15nM) and GD1b (20 nM) in 0.5 ml of Tris buffer saline (TBS) 10mM, pH 7.4 at 37°C for 20 min. Then NV-PLA<sub>2</sub> (2 μg) was added, final reaction volume was made up to 1 ml with TBS, pH 7.4 and incubated at 37°C for 60 min, washed with TBS, pH 7.4. The pellet obtained was used for determining Na<sup>+</sup>K<sup>+</sup>ATPase activity (Ames, 1966; Kaul and Krishnakanth, 1994; Moore, 1989). Pellet was dissolved and incubated in 0.5 ml of reaction mixture (Tris 50 mM, NaCl 350 mM, KCl 35 mM, MgCl<sub>2</sub> 7.5 mM, EDTA 0.5 mM, pH 7.0) for 10 min at 37°C. At the end of incubation period, ATP (15 mM) was added and further incubated at 37°C for 60 min. Reaction was stopped by adding 0.1ml of 10% TCA, kept on ice water for 10 min. The inorganic phosphorus liberated (Pi) was estimated according to the method of Fiske and Subbarao (1921). Prior to the assay parallel appropriate controls were maintained. The enzyme activity was expressed as μmole Pi released/mg membrane protein/hour.

Determination of Ca<sup>2+</sup>Mg<sup>2+</sup>ATPase activity

200 μg of membrane protein were preincubated with or without mixed gangliosides (18 nM), GM1 (15 nM) and GD1b (20 nM) in 0.5 ml of Tris buffer saline (TBS) 10mM, pH 7.4 at 37°C for 20 min. Then NV-PLA<sub>2</sub> (2 μg) was added, final reaction volume was made up to 1 ml with TBS, pH 7.4 and incubated at 37°C for 60 min, washed with TBS, pH 7.4. The pellet obtained was used for determining Na<sup>+</sup>K<sup>+</sup>ATPase activity (Ames, 1966; Kaul and Krishnakanth, 1994; Moore, 1989). Pellet was dissolved and incubated in 0.5 ml of reaction mixture (Tris 50 mM, NaCl 350 mM, KCl 35 mM, MgCl<sub>2</sub> 7.5 mM, EDTA 0.5 mM, pH 7.0) for 10 min at 37°C. At the end of incubation period, ATP (15 mM) was added and further incubated at 37°C for 60 min. Reaction was stopped by adding 0.1ml of 10% TCA, kept on ice water for 10 min. The inorganic phosphorus liberated (Pi) was estimated according to the method of Fiske and Subbarao (1921). Prior to the assay parallel appropriate controls were maintained. The enzyme activity was expressed as μmole Pi released/mg membrane protein/hour.
ml of Tris buffer saline (TBS) 10 mM, pH 7.4 at 37°C for 20 min. Then NV-PLA2 (2 µg) was added, final reaction volume was made up to 1 ml with TBS, pH 7.4 and incubated at 37°C for 60 min, washed with TBS, pH 7.4. The pellet obtained was used for determining Ca²⁺Mg²⁺ATPase activity (Ames, 1966; Kaul and Krishnakanth, 1994; Moore, 1989). Pellet was dissolved and incubated in 0.5 ml of reaction mixture (Imidazole 135 mM, MgCl₂ 5 mM, CaCl₂ 0.5 mM, pH 7.4) for 10 min at 37°C. At the end of incubation period, ATP (15 mM) was added and further incubated at 37°C for 60 min. Reaction was stopped by adding 0.1 ml of 10% TCA, kept on ice water for 10 min. The inorganic phosphorus liberated (Pi) was estimated according to the method of Fiske and Subbarao (Fiske and Subbarao, 1921). Prior to the assay parallel appropriate controls were maintained. The enzyme activity was expressed as µmole Pi released / mg membrane protein/hour.

Statistical analysis
All the values represents mean of triplicates and are expressed as Mean ± SD. The significance of the experimental observation was tested by student’s t – test (Snedecor and Cochran, 1976) and the values of p<0.05 were considered as significant.

RESULTS
N. naja venom was fractionated on a CM-Sephadex C-25 column by modifying the method of Bhat, MK and Gowda, TV (Bhat and Gowda, 1989). The enzyme elution profile showed the presence of PLA₂ activity in six peaks (Figure 1). Recovery of total PLA₂ activity from the CM-Sephadex C-25 column was 51% and the protein recovery was 88%. NV-PLA₂ (P-VII) accounted for 22% of the total protein recovered and 8% of the total PLA₂ activity recovered. The contaminating protein (<2%) found with NV-PLA₂ was removed by subjecting it to gel filtration on a Sephadex G-50 column. This fraction gave a single sharp Coomassie brilliant blue band on SDS-PAGE under both reduced and non-reduced conditions. The molecular weight estimated for NV-PLA₂ was 11500 by SDS-PAGE (Data not shown). NV-PLA₂ is slightly more toxic than crude venom with an Acute Mean Lethal Dose (AMD)₅₀ of 2.2 mg/kg body weight (i.p.) as compared to the AMD₅₀ of 2.6 mg/kg body weight for the whole venom.

PnA is fluorescent when partitioned into lipid environment and the emission of fluorescence disappears upon reaction of the membrane lipids with NV-PLA₂. The relative intensity of fluorescence from the membranes decreased after treating with NV-PLA₂ (Figure 2). NV-PLA₂ induced 72% damage to the membranes when compared to untreated membranes. The maximum damage was observed at 150th minute of incubation. Mixed gangliosides (18 nM) and GM1 (15 nM) prevented the decrease in the intensity of fluorescence and offered 81 and 86% protection respectively, whereas GD1b (20 nM) exhibited only 45% protection. GD1b did not significantly prevent the decrease in the intensity of fluorescence. EGTA (1mM), which was used as standard inhibitor, prevented the decrease in the intensity of fluorescence by 88%. Figure 3 show that Mixed gangliosides (18 nM) and GM1 (15nM) inhibited the NV-PLA₂ activity to 84% and 81% respectively, which was statistically significant, when compared to the group with
out any inhibitor. GD1b showed only 62% inhibition of the enzyme activity. In comparison to this, known inhibitor of PLA2 Dibromoace-tophenone (DBAP) at 35 nM inhibited the enzyme activity by 92.2%.

As shown in Table 1, activities of untreated erythrocyte membranes were 9.0 ± 0.38 and 9.7 ± 0.41 μmole/mg membrane protein/hour for Na\(^+\)K\(^+\) and Ca\(^{2+}\)Mg\(^{2+}\) ATPase respectively. Treatment of erythrocyte membrane with NV-PLA\(_2\) (2 μg) for 60 min at 37\(^o\)C resulted in significant inhibition of ATPase activities, and the activities were found to be 3.09 ± 0.19 and 4.58 ± 0.20 μmole/mg membrane protein/hour for Na\(^+\)K\(^+\) and Ca\(^{2+}\)Mg\(^{2+}\) ATPase respectively, when compared to untreated to membrane. Mixed gangliosides (18 nM) restored the Na\(^+\)K\(^+\) ATPase activity by 78%, whereas GM1 (15 nM) offered 74% restoration of the enzyme activity when compared to the NV-PLA\(_2\) treated membranes. GD1b (20 nM) did not show significant restoration (52%). The Ca\(^{2+}\)Mg\(^{2+}\) ATPase activity was restored by 80% and 81% with mixed gangliosides (18 nM) and GM1 (15 nM) respectively. GD1b (20 nM) showed only 50% restoration. α – Phenanthroline-an iron chelator at 100nM showed 91 and 89% restoration of Na\(^+\)K\(^+\) and Ca\(^{2+}\)Mg\(^{2+}\) ATPase activities.

**DISCUSSION**

Of the gangliosides tested, mixed gangliosides and GM1 were found to be effective inhibitors of NV-PLA\(_2\), restored Na\(^+\)K\(^+\) and Ca\(^{2+}\)Mg\(^{2+}\) ATPase activities and prevented erythrocyte membrane damage, when compared to GD1b, which did not prove to be an effective inhibitor. PLA\(_2\) has received considerable attention due to its involvement in a variety of different physiological and pathological functions (Dennis, 1994). PLA\(_2\) causes a complete degradation of glycerol phospholipids in the cell membrane. These enzymes attack lecithin-converting 2/3 of the phospholipids into lysoderivative. The above destruction of cell membrane integrity may in turn lead to phospholipase activation and release of arachidonate followed by the biosynthesis and release of eicosanoids to surrounding tissue, which, by themselves cause considerable membrane damage (Condrea et al., 1980; Kannagi et al.,...
1981; Mukherjee et al., 1998). In this context, an inhibition PLA2 enzyme plays a significant role in preventing membrane damage.

In the present study, mixed gangliosides and GM1 inhibited NV-PLA2 activity in a concentration dependent manner. A maximum inhibition of the NV-PLA2 activity was achieved at 18 nM and 15 nM of mixed gangliosides and GM1 respectively (Figure 3). Since gangliosides are ubiquitous membrane components in the outer leaflet of lipid bilayer, they serve as receptor for many toxins of bacterial origin, especially GM1, the specific receptor for cholera toxin (Orlandi and Fishman, 1993), which in turn inhibit the toxin action. They are found to play an important role in regulating PLA2 activity against dipalmityloleoylphosphatidylcholine in small unilamellar bilayer and mixed monolayers (Maggio et al., 1991; Mellan-by and Pope, 1976). The inhibition of NV-PLA2 by mixed gangliosides and GM1 could be attributed to the modulation of the catalytic activity of PLA2 at the interface itself, beyond the initial steps of enzyme adsorption and activation, probably through modifications of the intermolecular organization of the membrane components. It is well known that snake venoms cause cell mem-brane asymmetry by degradation of glycerol phospholipids of the membranes. The most common method for assessing membrane damage is to determine the activities of membrane bound ion transport ATPases such as Na+K+ and Ca2+Mg2+ ATPases. In this study along with determining Na+K+ and Ca2+Mg2+ ATPases activities, we employed another quantitative method using PnA (McKenna, 1991). When PnA was used the relative intensity of fluorescence of the erythrocyte membrane decreased on treatment with NV-PLA2 indicating alteration in the membrane organization thus leading to increased porosity of the membrane enabling the fluorescent probe PnA to penetrate more.

The results in Figure 2 indicate that mixed gangliosides and GM1 were able to prevent the decrease in the relative intensity of fluorescence when compared to GD1b. EGTA a well known chelator of Ca2+ ions showed maximum prevention, which could be due to chelation of Ca2+ ions, since these Ca2+ ions are essential cofactors for most of the toxic enzymes of snake venom such as PLA2, sphingomyelinases and so on. Since, gangliosides also exhibit high specificity for these divalent cations (Kannagi et al., 1981), the protection offered by gangliosides could be due to initial inhibition of toxic PLA2 enzyme and chelation of divalent metal ions required by the toxic venom enzymes. At the cellular level of organization, the flux of ions across membranes is largely dependent on transmembrane ion specific proteins such as sodium – potassium dependent ATPases.

The Na+K+ ATPase, an integral part of plasma membrane is responsible for the control of sodium and potassium transport (Cohly et al., 2003). Low intracellular calcium is maintained by CaM – activated Ca2+ pump ATPase located in the plasma membrane (Logon-Smith et al., 2002). Exposure of the erythrocyte membranes to NV-PLA2 significantly decreased the Na+K+ and Ca2+Mg2+ ATPases activities (Table 1), thereby altering the ionic gradients, disorganization of the mem-brane lipid bilayer and eventually cell death. Mixed gangliosides and GM1 effectively restored the Na+K+ and Ca2+Mg2+ ATPases activities of the erythrocyte membrane when compared to the membranes treated with NV-PLA2.

In conclusion, exogenous treatment of mixed gangliosides and GM1 could effectively modulate NV-PLA2 induced erythrocyte membrane damage and significantly inhibit the enzyme activity when compared to ganglioside GD1b. The above scenario then means that mixed gangliosides and GM1 could act as receptors for the toxic venom components, which in turn play a significant role in inhibition of NV-PLA2. Ganglioside GD1b did not show to be an effective inhibitor. This could be due to structural differences between GM1 and GD1b. However investigations concerning the mechanism of inhibition of NV-PLA2 induced toxic effect on erythrocyte membrane by mixed gangliosides and GM1 is in progress. Studies in relation to understanding the mode of interaction between NV-PLA2 and gangliosides could give a better indication of how gangliosides inhibit the toxicity of NV-PLA2. The data obtained from the above study prompts to investigate in future the potential effect of gangliosides in neutralizing the lethality of toxic NV-PLA2 in the in vivo system. Hence, substantial evidence from in vivo experiments could make gangliosides as novel, potential therapeutic agent for treating snake bite victims.

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