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Guanidination of notexin promotes its phospholipase A_2 activity-independent fusogenicity on vesicles with lipid-supplied negative curvature

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ABSTRACT

To address the requirement of phospholipase A_2 (PLA_2) activity in membrane fusion events and membrane perturbation activity of notexin and guanidinated notexin (Gu-notexin), the present study was conducted. Notexin and Gu-notexin did not show PLA_2 activity after the removal of Ca^{2+} with EDTA. Metal-free notexin and Gu-notexin were found to induce membrane leakage and fusion of phospholipid vesicles. Fusogenic activity of native and modified notexin correlated positively with their membrane-damaging activity underlying the deprivation of PLA_2 activity. Compared with Ca^{2+}-bound Gu-notexin, fusogenicity of metal-free Gu-notexin was notably increased by incorporation of cholesterol, cholesterol sulfate, phosphatidylethanolamine, α-tocopherol and phosphatidic acid that supplied negative curvature into phospholipid bilayer. The ability of Gu-notexin to induce membrane fusion of vesicles with lipid-supplied negative curvature was higher than that of notexin regardless of the absence or presence of Ca^{2+}. Consistently, metal-free Gu-notexin markedly induced membrane fusion of red blood cells (RBCs) compared with metal-free notexin, and fusion activity of metal-free Gu-notexin on cholesterol-depleted RBCs notably reduced. Compared with notexin, Gu-notexin highly induced uptake of calcein-loaded phosphatidylcholine (PC)/cholesterol and PC/cholesterol sulfate vesicles by K562 cells in the presence of EDTA. Taken together, our data suggest that notexin and Gu-notexin could induce vesicle leakage and fusion via a PLA_2 activity-independent mechanism, and guanidination promotes PLA_2 activity-independent fusogenicity of notexin on vesicles with lipid-supplied negative curvature.

1. Introduction

Notexin, a presynaptic phospholipase A_2 (PLA_2) neurotoxin purified from the venom of *Notechis scutatus scutatus*, is a basic protein with 119 amino acids and 7 disulfide bridges (Westerlund et al., 1992). Besides its presynaptic neurotoxicity, which blocks neuromuscular transmission by affecting the release of acetylcholine, notexin exhibits PLA_2 activity and myotoxicity (Rigoni et al., 2004; Plant et al., 2006). Notexin belongs to Elapidae snake venom group IA PLA_2 and is structurally homologous to mammalian pancreatic group IB PLA_2 (Westerlund et al., 1992). Given that presynaptic PLA_2

Abbreviations: Chol, Cholesterol; CS, Cholesterol sulfate; CD, Circular dichroism; DMPA, Dimyristoyl phosphatidic acid; DHTPC, Dihepanoyl-thio-phosphatidylcholine; Laurdan, 6-Dodecanoyl-2-dimethyl-amino-naphthalene; EYPC, Egg yolk phosphatidylcholine; EYPE, Egg yolk phosphatidylethanolamine; EYSM, Egg yolk sphingomyelin; Gu, Guanidinated; HNB-Br, 2-Hydroxy-5-nitrobenzyl bromide; Rh-PE, Lissamine rhodamine B 1,2-dihexadecanoly-*sn*-glycero-3-phosphoethanolamine; MβCD, Methyl-β-cyclodextrin; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine; PLP, Pyridoxal-6′-phosphate; RBC, Red blood cell; TFA, Trifluoroacetic acid; TNP, Trinitrophenylated; TNBS, Trinitrobenzene sulfonic acid.

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neurotoxins show both PLA_2 activity and presynaptic neurotoxicity (Pungercar and Krizaj, 2007; Montecucco et al., 2008), it is important to clarify the role of enzymatic activity in inducing the toxic effect. There is considerable evidence in favor of the essential role of PLA_2 activity in presynaptic neurotoxicity (Rigoni et al., 2005; Paoli et al., 2009), but quantitative relationship between PLA_2 activity and pharmacological activity has not been clearly proved (Rosenberg, 1997; Rigoni et al., 2004; Paoli et al., 2009). Thus, it is no doubt that the PLA_2 activity is involved in the pharmacological activities of presynaptic neurotoxins. However, several lines of evidence do not exclude possible addition non-PLA_2 mediated effect (Rosenberg, 1997).

Our recent studies indicated that notexin and guanidinated notexin (Gu-notexin) induced calcein leakage from phosphatidylcholine/sphingomyelin/cholesterol vesicles underlying the deprivation of PLA_2 activity (Kao et al., 2010a). This suggests that notexin and Gu-notexin may cause membrane damage via the catalytic activity-independent manner. Rufini et al. (1992) found that *Vipera ammodytes ammodytes* ammodytin L and *Bothrops asper* myotoxin L, structurally homologous with PLA_2 enzymes, induced membrane leakage and membrane fusion of phosphatidylcholine (PC)/phosphatidic acid (PA) vesicles in a Ca^{2+}-independent manner. Moreover, peptide-induced vesicle–vesicle fusion caused leakage of vesicular contents simultaneously (Martin and Ruysschaert, 1997; Villar et al., 2000; Samsonov et al., 2002; van den Bogaart et al., 2007). Thus, it is possible that notexin and Gu-notexin induce membrane fusion without the involvement of PLA_2 activity. To address this problem, the present study was conducted.

2. Materials and methods

Notexin was purified from *Notechis scutatus scutatus* venom according to the procedure described previously (Chang, 1996). Calcein, cholesterol, cholesterol sulfate (CS), dimyristoyl phosphatidic acid (DMPA), egg yolk phosphatidylcholine (EYPC), egg yolk phosphatidylethanolamine (EYPE), egg yolk sphingomyelin (EYSM), sodium dithionite, methyl-β-cyclodextrin (MβCD) and α-tocopherol were purchased from Sigma–Aldrich Inc. Calcein-AM, Lissamine rhodamine B 1,2-dihexadecanoly-*sn*-glycero-3-phosphoethanolamine (Rh-PE), N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2- dihexadecanoyl-*sn*-glycerol- 3-phosphoethanolamine (NBD-PE) and 6-dodecanoyl-2-dimethyl-aminonaphthalene (Laurdan) were products of Molecular Probes. Sepharose 6B was obtained from Amersham Biosciences. All other reagents were products of analytical grade.

2.1. Chemical modification of notexin

Modification of notexin with O-methylisourea, trinitrobenzene sulfonic acid (TNBS), pyridoxal-5′-phosphate (PLP), and 2-hydroxy-5-nitrobenzyl bromide (HNB-Br) were conducted essentially according to the procedure described previously (Yang and Chang, 1984, 1990; Chang, 1996). Trp residues at positions 20 and 110 were modifed by HNB-Br (Yang and Chang, 1984), and HNB-notexin was further purified by HPLC on a SynChropak RP-P column (4.6 mm × 25 cm)that equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of 10%–50% acetonitrile for 70 min. The flow rate was 0.8 ml/min, and the eluate was monitored at 280 nm. The results of amino acid analyses showed that both Trp20 and Trp110 of notexin were modified by HNB-Br. Guanidinated notexin (Gu-notexin) was prepared by modification of Lys residues with O-methylisourea. Trinitrophenylation (TNP) of Gu-notexin with TNBS led to selective incorporation of TNP group onto Asn-1, and TNP-Gu-notexin was purified by HPLC on a SynChropak RP-P column (Yang and Chang, 1990). The N-terminus of notexin was selectively modified by TNBS, and trinitrophenylated notexin (TNP-notexin) was purified by SynChropak RP-P column (Yang and Chang, 1990). Notexin, Gu-notexin, TNP-Gu-notexin, TNP-notexin and HNB-notexin were rechromatographed on a SynChropak RP-P column under the same conditions as described above. It was found that their biological activities remained unchanged. This indicates that separation of the modified derivatives with an acetonitrile gradient did not cause irreversible changes in their biological activities. Lys residues at positions 82 and 115 were modified with pyridoxal-5′-phosphate (PLP), the resulting PLP-notexin was purified by Protein-Pak SP-8HR column (Chang, 1996). To verify the purity of modified derivatives, Gu-notexin, TNP-Gu-notexin, TNP-notexin, PLP-notexin and HNB-notexin were applied on a SynChropak RP-P column that equilibrated with 0.1% TFA and eluted with a linear gradient of 10%–50% acetonitrile for 70 min (Fig. 1). Moreover, the result of native polyacrylamide gel electrophoresis revealed that modified derivatives of notexin were homogeneous (data not shown).

2.2. Determination of PLA_2 activity

PLA_2 activity of notexin and modified derivatives was measured spectrophotometrically using the PLA_2 activity

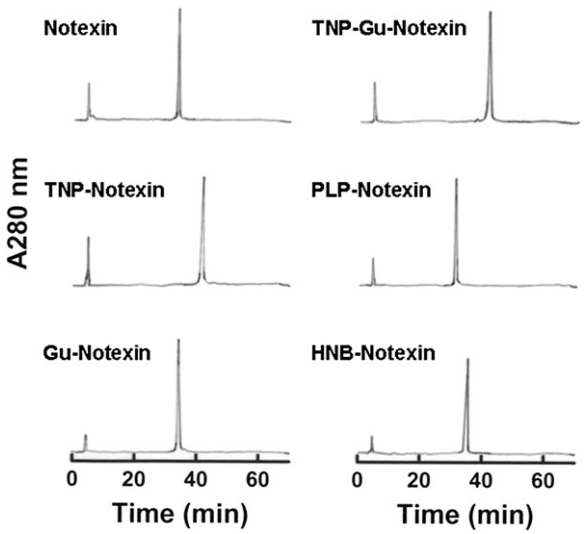

Fig. 1. Chromatographic analyses of native and modified notexin using a HPLC column. Native and modified notexin were applied on a SynChropak RP-P column (4.6 mm × 25 cm) equilibrated with 0.1% TFA and eluted with a linear gradient of 10–50% acetonitrile for 70 min. The flow rate was 0.8 ml/min.