Biochemical Pharmacology, Vol. 44, No. 6, pp. 1073–1083, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

EFFECTS OF SNAKE VENOM PHOSPHOLIPASE A_2 TOXINS (β-BUNGAROTOXIN, NOTEXIN) AND ENZYMES (*NAJA NAJA ATRA, NAJA NIGRICOLLIS*) ON AMINOPHOSPHOLIPID ASYMMETRY IN RAT CEREBROCORTICAL SYNAPTOSOMES

ANNAHITA GHASSEMI* and PHILIP ROSENBERG†
Section of Pharmacology and Toxicology, The University of Connecticut, School of Pharmacy, Storrs, CT 06269-2092, U.S.A.

(*Received* 7 *January* 1992; *accepted* 22 *May* 1992)

Abstract—The effects of snake venom phospholipase A_2 (PLA_2) toxins (β-bungarotoxin, notexin) and PLA_2 enzymes (*Naja nigricollis, Naja naja atra*) on aminophospholipid asymmetry in rat cerebrocortical synaptic plasma membranes (SPM) were examined. Incubation of intact synaptosomes with 2 mM 2,4,6-trinitrobenzene sulfonic acid (TNBS) for 40 min, under non-penetrating conditions, followed by SPM isolation, allowed us to calculate the percentage of phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the outer leaflet of the SPM, while incubation with disrupted synaptosomes provided total labeling values with the difference representing labeling of the inner leaflet. We found that 30% of the PE and 2% of the PS were in the outer leaflet, with 54% of the PE and 80% of the PS in the inner leaflet; 16% of the PE and 18% of the PS was inaccessible to TNBS. PLA_2 toxins and enzymes increased in a concentration-dependent manner the percentage of PS and, to a lesser extent, the percentage of PE in the outer leaflet of the SPM, due to a redistribution from the inner to the outer leaflet. There was no correlation between the PLA_2 enzymatic activities and the increased percentage of PS in the outer leaflet of the SPM induced by the PLA_2 toxins and enzymes. Alteration of aminophospholipid asymmetry does not explain the greater presynaptic specificity and potencies of the PLA_2 toxins as compared to the PLA_2 enzymes, but may be associated with the increased acetylcholine release from synaptosomes induced by both the toxins and enzymes.

β-Bungarotoxin (β-BuTX‡), isolated from *Bungarus multicinctus* snake venom [1], and notexin, isolated from *Notechis scutatus scutatus* snake venom [2], have phospholipase A_2(PLA_2) activity, act specifically at the presynaptic motor nerve terminals to increase and then block acetylcholine (ACh) release [1, 3–7], and are referred to as "PLA_2 toxins". In the CNS, they alter neurotransmitter release from synaptosomes as well as block choline and Ca^{2+} uptake into the synaptosomes [8–13]. Despite their high lethal activity and potent presynaptic actions, β-BuTX and notexin have relatively weak PLA_2 enzymatic activities upon artificial substrates [14, 15]. On the other hand, other PLA_2 enzymes, such as these isolated from the venoms of the snakes *Naja naja atra* [16] and *Naja nigricollis* [17], which have relatively low lethal potencies and are not specific in their actions for presynaptic sites [14, 15, 18], have higher PLA_2 enzymatic activities using artificial substrates than β-BuTX and notexin [15, 19, 20] and are referred to as "PLA_2 enzymes". These PLA_2 enzymes, however, do have a wide variety of biological effects including actions on both pre- and postsynaptic membranes (heart, neuromuscular junction) as well as on non-excitable tissues (hemolytic and anticoagulant activity) [14, 15, 18]. However, despite differences in potencies and specificities of action, both those proteins called PLA_2 toxins and those proteins called PLA_2 enzymes hydrolyze membranal phospholipids, including those in synaptosomes, and alter neurotransmitter release, both in the peripheral and central nervous system [1–21].

The asymmetric distribution of phospholipids across the bilayer of various biological membranes is well established [22–26]. Phosphatidylcholine (PC) and sphingomyelin are predominantly found in the outer leaflet while phosphatidylserine (PS) and the majority of phosphatidylethanolamine (PE) are in the inner leaflet of the membrane. The asymmetry of the aminophospholipids (PE, PS) across the lipid bilayer is thought to be maintained by two mechanisms. One mechanism involves a strong interaction between the aminophospholipids and cytoskeletal proteins such as spectrin and protein

* Current address: Proctor & Gamble Co., Cincinnati, OH 45217, U.S.A.

† Corresponding author: Prof. P. Rosenberg, Section of Pharmacology and Toxicology, The University of Connecticut, School of Pharmacy, U-92, Storrs, CT 06269-2092, U.S.A. Tel. (203) 486-2213; FAX (203) 486-4998.

‡ Abbreviations: SPM, synaptic plasma membrane; β-BuTX, β-bungarotoxin; PLA_2 (EC 3.1.1.4), phospholipase A_2; ACh, acetylcholine; TNBS, 2,4,6-trinitrobenzene sulfonic acid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LPS, lysophosphatidylserine; PE, phosphatidylethanolamine; TNP-LPS, trinitrophenylated lysophosphatidylserine; TNP-PS, trinitrophenylated phosphatidylserine; TNP-LPE, trinitrophenylated lysophosphatidylethanolamine; and TNP-PE, trinitrophenylated phosphatidylethanolamine.

4.1 in the red blood cells and synapsin in the brain [27–33]. The other mechanism involves an ATP-dependent aminophospholipid translocase which catalyzes the outward–inward transbilayer movement of PE and PS [33–36].

Alterations in aminophospholipid asymmetry could result in changes in the functioning of cell membranes. For example, the increased exposure of PS at the outer surface of the platelet membrane upon activation is correlated with the blood coagulating action of the platelets [26, 28, 33]. In irreversibly sickled red blood cells in which there is altered PE and PS asymmetry, there is enhanced blood clotting and a greater ability to adhere to endothelial cells [33, 37, 38]. In addition, alterations in PE asymmetry in the synaptic plasma membrane (SPM) have been correlated with changes in potassium-stimulated dopamine release [39]. The redistribution of PE or PS to the outer leaflet of the membrane could be due to disruption of the interaction between the aminophospholipids of the plasma membrane and cytoskeletal proteins and/or inhibition of aminophospholipid translocase activity. It has been shown in our laboratory [40] that the PLA_2 toxins and enzymes inhibit phosphorylation of cytoskeletal proteins including synapsin.

Because changes in aminophospholipid asymmetry have been associated with alterations in physiological function, it was of interest to study the effects of the PLA_2 toxins and PLA_2 enzymes on aminophospholipid asymmetry in the SPM. In addition, we attempted to determine whether phospholipid hydrolysis was essential for the changes in the asymmetry of PE and PS which we observed following exposure to the toxins and enzymes. In these studies, we used 2,4,6-trinitrobenzene sulfonic acid (TNBS) which has been widely used as an impermeable probe to study aminophospholipid asymmetry across the SPM bilayer [24, 39, 41, 42]. TNBS covalently binds, under nonpenetrating conditions, to the amino groups of PE and PS in the outer leaflet of the synaptosomes [24, 42, 43]. This is the first study to examine the effects of PLA_2 toxins (β-BuTX, notexin) and enzymes (*N. nigricollis, N. n. atra*) on aminophospholipid asymmetry in the rat brain SPM.

MATERIALS AND METHODS

Materials. Lyophilized *B. multicinctus*, *N. nigricollis* and *N. s. scutatus* snake venoms were purchased from Miami Serpentarium Laboratories (Salt Lake City, UT); *N. n. atra* snake venom was freshly collected and lyophilized in Kaohsuing, Taiwan, by Dr. C-C. Yang. The most basic and toxic PLA_2 (CMS-9, pI 10.6) from *N. nigricollis* venom, the acidic PLA_2 (major enzyme, pI 5.2) from *N. n. atra* venom, β-BuTX and notexin were all isolated and purified by Dr. C-C. Yang (National Tsing Hua University, Hsinchu, Taiwan) as previously described [16, 44]. Homogeneities of the toxins and enzymes as determined by amino acid analysis and electrophoresis have been described [2, 16, 44, 45]. Silica gel (Kieselgel 60 Hr) was purchased from EM Science (Cherry Hill, NJ). Ficoll (type 400-DL), fatty acid free bovine serum albumin (prepared from fraction V albumin), sodium bicarbonate, Tris–HCl and trinitrophenylated phosphatidylethanolamine (TNP-PE) were purchased from the Sigma Chemical Co. (St. Louis, MO). TNBS was obtained from Molecular Probes Inc. (Eugene, OR). All other reagents were of analytical grade.

Preparation of synaptosomes and synaptic plasma membranes. Male Sprague–Dawley rats (200–300 g) were obtained from Charles River Laboratories, Wilmington, MA. After decapitation, the brains were quickly removed and the cerebral cortex was dissected. Each group of synaptosomes was prepared from cerebral cortices (approximately 3.0 g wet tissue) of five rats. The dissected tissues were homogenized (20%, w/v), using a Dounce-type glass homogenizer, in cold isolation medium consisting of 0.32 M sucrose, 10 mM Tris–HCl and 10 mM EDTA (pH 7.4). All subsequent steps were performed on ice. Synaptosomes were isolated from the homogenate using a Ficoll-sucrose discontinuous gradient centrifugation [46]. SPM were isolated following osmotic lysis of synaptosomes and purified using discontinuous sucrose gradient centrifugation [47]. Purification of our synaptosomal and SPM preparations was confirmed by electron microscopy and by the following enzyme markers: cytochrome oxidase for mitochondria [48], lactate dehydrogenase for cytosolic fractions [49], and Na^+/K^+-ATPase for plasma membrane [50].

Aminophospholipid asymmetry studies. In preliminary experiments, we determined the concentrations and incubation times for TNBS which would label all accessible pools of PE and PS on the outer leaflet without disrupting synaptosomal membrane integrity. Procedures similar to those reported in the literature and shown to be optimal were used [24, 42]. Synaptosomes were exposed to 0.5 to 6 mM TNBS dissolved in a 173 mM $NaHCO_3$ buffer (pH 8.5) containing 11 mM dextrose. The pH of the reaction medium must be alkaline since TNBS only reacts with deprotonated amines [43]. A pH of 8.5 is most commonly used with TNBS and does not disrupt the membrane or increase permeability of synaptosomes [24] or erythrocytes [51]. The incubations were carried out for 5–120 min at 4° in the dark since at this low temperature TNBS penetration would be minimal [52], and since during formation the trinitrophenylated phosphatidylserine (TNP-PS) product is light-sensitive [23]. After terminating the reaction by the addition of an acidified (pH 6.7) $NaHCO_3$ buffer and centrifugation of the suspension, synaptosomes were washed once to ensure removal of the excess TNBS. Synaptosomes were then lysed, and SPM were isolated and processed for phospholipid analysis.

The effect of PLA_2 treatment on aminophospholipid asymmetry was assessed in isolated synaptosomes, suspended in a physiological buffer (pH 7.4) of the following mM concentration: NaCl, 133; KCl, 3.5; $NaH_2PO_4 \cdot 2H_2O$, 0.5; $NaHCO_3$, 26; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2$, 2.5; urea, 3.3. The synaptosomal suspensions were incubated with buffer alone or with 0.5 or 5 nM concentrations of the PLA_2 toxins or PLA_2 enzymes or with 50 nM β-BuTX for 15 min at 37°. This incubation time with these concentrations of the PLA_2 toxins and enzymes

did not disrupt synaptosomal membrane integrity as determined by measuring the release of [^{3}H]*d*-glucose-6-phosphate from synaptosomes [21; unpublished results]. [^{3}H]*d*-Glucose-6-phosphate has a molecular weight similar to that of TNBS and has been widely used as a marker to determine synoptosomal and cell membrane integrity [53, 54]. In addition, the synaptosomes were exposed for 15 min to 50 nM notexin; however, this treatment did disrupt membranal permeability as assayed by the above noted method [21]. The incubation was terminated in both control and treated preparations by the addition of 10 mM EDTA (in the above buffer solution but without Ca^{2+}) and a 5-min centrifugation of the synaptosomes. The synaptosomal pellets were then resuspended and incubated in 2 mM TNBS in $NaHCO_3$ buffer (pH 8.5) as described above, for 40 min, followed (after washing to ensure removal of excess TNBS) by isolation of the SPM. To determine whether phospholipid hydrolysis was essential for the effects of PLA_2 toxins and enzymes on aminophospholipid asymmetry, synaptosomes were incubated for 15 min with 5 nM PLA_2 toxins and enzymes in the absence of extracellular Ca^{2+} and in the presence of EDTA (10 mM) to chelate any residual Ca^{2+}. Phospholipid hydrolysis measurements were made on the same samples upon which aminophospholipid asymmetries were measured.

To determine the total accessible pool of aminophospholipids in the inner plus the outer leaflets of the SPM, the synaptosomes were lysed prior to exposure to the TNBS. Synaptosomes were lysed by osmotic shock with 6 mM Tris–HCl buffer (pH 8.2) for 90 min at 4° with occasional sonication followed by freezing and thawing to ensure complete membrane disruption.

Phospholipid analysis. Lipids were extracted from the SPM with chloroform:methanol 1:3 [55], followed by 2:1 [56]. Phospholipids, lysophospholipids, and trinitrophenylated-aminophospholipids were separated by two-dimensional thin-layer chromatography (Fig. 1) of the lipid extracts [57]. The silica gel HR plates were developed in the first dimension with chloroform–methanol–water (130:50:8) and in the second dimension with heptanone–acetic acid–water (80:60:10.5) [57]. The silica gel spots were removed by scraping and the lipid phosphorus content was determined spectrophotometrically [58]. The TNP-PE and TNP-PS products were yellow in color and migrated to different locations than the unreacted PE and PS and other phospholipids (Fig. 1). The other phospholipids and neutral lipids were visualized by iodine vapor. The migration of TNP-PE and TNP-PS was verified by comparison with trinitrophenylated standards purchased from the Sigma Chemical Co. and with PE (L-α-phosphatidylethanolamine, dipalmitoyl), and PS (L-α-phosphatidylserine) which were trinitrophenylated with 2 mM TNBS solution at 4° in the laboratory. The percent phospholipid hydrolysis was calculated by dividing the lipid phosphorus content of the lysophospholipid spot by the total phosphorus content of the lyso- plus the parent phospholipid spot and multiplying by 100. The percent phospholipid labeled with TNBS was

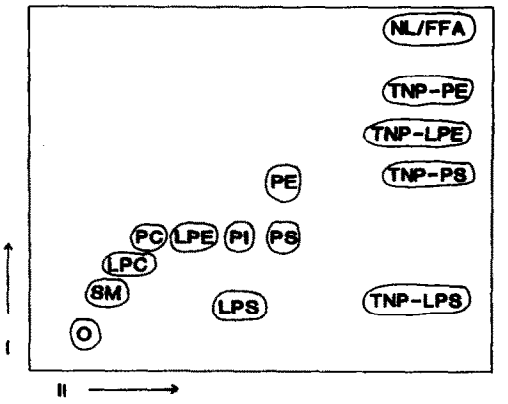

Fig. 1. Two-dimensional thin-layer chromatographic separation of phospholipids, lysophospholipids and trinitrophenylated (TNP) phospholipid derivatives. Approximately 30 μg of phosphorus was spotted at the lower left corner of the TLC plate (origin). The plates were developed in two dimensions: chloroform:methanol:water (130:50:8) (I) and heptanone:glacial acetic acid:water (80:60:10.5) (II). After development in the first dimension the plates were heated at 60° for 30 min before being developed in the second dimension. Abbreviations: O, origin; LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; TNP-LPS, trinitrophenylated lysophosphatidylserine; TNP-PS, trinitrophenylated phosphatidylserine; TNP-PE, trinitrophylated phosphatidylethanolamine; TNP-LPE, trinitrophenylated lysophosphatidylethanolamine; NL, neutral lipids; and FFA, free fatty acids.

calculated by dividing the lipid phosphorus content of the TNP-phospholipid (PE or PS) spot by the total lipid phosphorus content of the TNP-phospholipid spot plus the unreacted phospholipid (PE or PS) spot and multiplying by 100. Unlike PE and PS, lysophosphatidylethanolamine (LPE) and lysophosphatidylserine (LPS) produced by the PLA_2 toxins and enzymes in the SPM were not labeled by TNBS. Since we used non-penetrating and non-disrupting concentrations of the PLA_2 toxins and PLA_2 enzymes (except for experiments with 50 nM notexin) [21], the lyso products probably originated from phospholipid hydrolysis in the outer monolayer of the plasma membrane bilayer, rather than from the inner monolayer, although this has not been specifically determined. Therefore, in experiments in which PLA_2 toxins and enzymes were used, the total percentage of PE and PS in the outer leaflet of the bilayer was calculated by adding the percentage of LPE or LPS to the percentage of PE or PS labeled by TNBS.

Statistical analysis. All data are presented as means ± SEM with an $N > 3$. Differences between the mean values were compared by analysis of variance. If statistically significant differences were found, a Duncan's multiple range test was used for comparison between control and treated samples. A P value of <0.05 was considered as the level of significance.

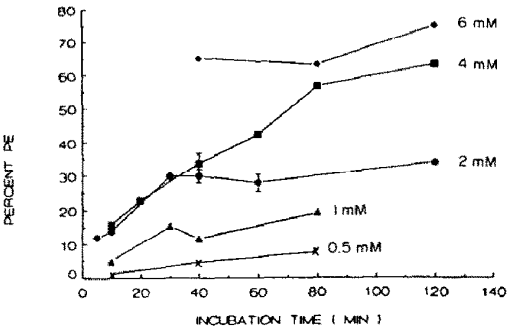

Fig. 2. Time-course of TNBS-labeling of PE in SPM following incubation of intact cerebrocortical synaptosomes with TNBS. Synaptosomes were exposed to 0.5, 1, 2, 4 and 6 mM TNBS for 5, 10, 20, 30, 40, 60, 80 or 120 min in a 173 mM $NaHCO_3$ buffer (pH 8.5) containing 10 mM dextrose at 4°, in the dark with shaking. Following lysis of the synaptosomes, SPM were isolated. Each point is the mean of 2–6 determinations. The points with $N > 3$ are means ± SEM. The points with 2 determinations had ranges of ±10% or less.

The statistical analysis was run under the release of 82.2B of SAS (SAS Institute Inc., Cary, NC) at The University of Connecticut.

RESULTS

An important requirement for the reliability of aminophospholipid asymmetry studies is that TNBS should only label PE and PS on the outer leaflet of the synaptic membrane without disrupting synaptosomal membrane integrity, which would expose phospholipid on the inner leaflet. Therefore, experiments were initially performed to determine the conditions under which TNBS could be used in intact synaptosomes as an impenetrable probe. A plateau in the labeling of PE would be expected once the entire pool of PE on the outer leaflet is labeled with TNBS. However, little labeling of PS by TNBS in intact synaptosomes would be expected since the great majority of the PS is on the inner leaflet of the SPM [24]. To determine optimal conditions, the concentration of TNBS (0.5–6 mM) and incubation period (5–120 min) were varied and the percentage of PE labeled in the SPM by TNBS was calculated (Fig. 2). TNBS appeared to react with all accessible aminophospholipids in the outer leaflet of the plasma membrane in the synaptosomes at a concentration of 2 mM and an incubation time of 40 min since under these conditions a plateau in the labeling of PE (30 ± 2%) was reached with no change in labeling between 30 and 120 min (Fig. 2), suggesting labeling of all accessible aminophospholipids. Furthermore, 2 mM TNBS at all incubation times tested (30, 40, 80, and 120 min) showed minimal labeling of PS (2 ± 2%; $N = 3$–6), suggesting that the entire PS is located in the inner leaflet of the SPM. Incubation with 2 mM TNBS for 40 min did not cause any marked alteration in the electron microscopic appearance of the synaptosomes (results not shown). Higher concentrations of TNBS (4 and 6 mM) were not suitable since no plateau in labeling (4 mM) and very high levels of labeling (4 and 6 mM) were observed. Furthermore, 4 and 6 mM TNBS (40, 60, 80 and 120 min) labeled 30–40% of PS in the intact synaptosomes ($N = 2$–6; results not shown). In addition, incubation with 6 mM TNBS for 40 min caused disruption of the SPM and mitochondria as judged by electron microscopy. These results indicated that 4 and 6 mM concentrations of TNBS penetrated into the synaptosomes and labeled some of the PE and PS that were on the inner leaflet of the SPM. In contrast, the labeling of PE by 0.5 and 1 mM concentrations of TNBS in the incubation times studied was much lower than the plateau reached with 2 mM TNBS (Fig. 2).

In the next series of experiments, the asymmetry of PE and PS across the SPM bilayer was confirmed. Three pools of aminophospholipids were found in the SPM: outer leaflet, inner leaflet and inaccessible pool. The percentage of aminophospholipids in the outer leaflet of the plasma membrane was determined by measuring the percent labeling of these phospholipids in SPM isolated from intact synaptosomes exposed to TNBS under non-penetrating conditions. Therefore, 30 ± 2% PE and 2 ± 2% PS labeled by TNBS (2 mM, 40 min) represented the percentages of PE and PS present in the outer leaflet of the SPM. By exposing lysed synaptosomes to 2 mM TNBS for 40 min at 4° in the dark, we found that the total labeling of PE and PS (both outer and inner leaflets) in the SPM was 84 and 82%, respectively. These results indicated that 54% (84 − 30%) of the PE and 80% (82 − 2%) of the PS are located in the inner leaflet of the SPM. Sixteen percent (100 − 84%) of the PE and 18% (100 − 82%) of the PS did not react with TNBS under conditions in which TNBS would be expected to be permeable to the membrane and is, therefore, considered to be inaccessible.

To determine the effects of the PLA_2 toxins and PLA_2 enzymes on aminophospholipid asymmetry, the intact synaptosomes were exposed to 0.5 and 5 nM PLA_2 toxins and enzymes and 50 nM PLA_2 toxins for 15 min (37°) prior to the exposure to TNBS. β-BuTX and notexin at 0.5 and 5 nM concentrations had no significant effects on the percentage of PE labeled while 50 nM concentrations significantly ($P < 0.05$) increased the percentage of PE exposed on the outer leaflet of the SPM (Fig. 3). At a 0.5 nM concentration, the PLA_2 enzymes had no significant effects on the percentage of PE exposed, while at the 5 nM concentration they significantly increased the percentage of PE on the outer leaflet of the SPM (Fig. 3). The effects of the PLA_2 toxins and PLA_2 enzymes on the percentage of PS labeled in the outer leaflet of the SPM are shown in Fig. 4. All concentrations caused significant increases ($P < 0.05$) in the percentage of PS labeled in the SPM. Removal of extracellular Ca^{2+} and addition of 10 mM EDTA did not alter significantly the effects of 5 nM β-BuTX, notexin or *N. nigricollis* PLA_2 on PS asymmetry; *N. n. atra* was not tested (Fig. 5).

The PLA_2 toxins and PLA_2 enzymes caused concentration-dependent increases in the hydrolysis

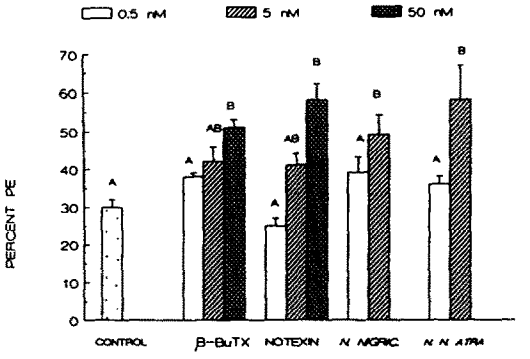

Fig. 3. Effects of PLA_2 toxins and PLA_2 enzymes on the percentage of PE in the outer leaflet of rat cerebrocortical SPM. Isolated intact synaptosomes were exposed to 0.5 or 5 nM PLA_2 toxins and enzymes or 50 nM PLA_2 toxins for 15 min at 37° prior to TNBS labeling. Means ± SEM (N = 3–6) are shown. For each concentration of PLA_2 the means show the same letters indicating no significant differences between the treatments ($P > 0.05$). Letters are also used to compare all concentrations of PLA_2 treatments with the control value; means with the letter B were significantly ($P < 0.05$) different from the control value (letter A).

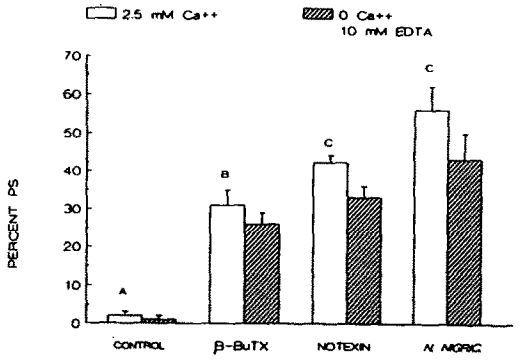

Fig. 5. Effect of calcium removal and EDTA addition on the increased PS in the outer leaflet of SPM, upon exposure to PLA_2 toxins and PLA_2 enzyme. Intact cerebrocortical synaptosomes were incubated with 5 nM PLA_2 toxins and *N. nigricollis* PLA_2 in the presence of 2.5 mM Ca^{2+} (normal condition) or 0 Ca^{2+} + 10 mM EDTA, prior to exposure to 2 mM TNBS. Each column and bar represent mean ± SEM (N = 3–6). In the presence of Ca^{2+}, means with different letters were significantly different ($P < 0.05$). The 0 Ca^{2+} plus 10 mM EDTA results were not significantly different ($P > 0.05$) from their corresponding 2.5 mM Ca^{2+} results.

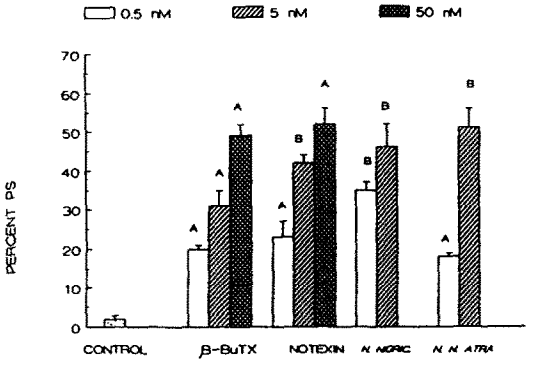

Fig. 4. Effects of PLA_2 toxins and PLA_2 enzymes on the percentage of PS in the outer leaflet of rat cerebrocortical SPM. Isolated intact synaptosomes were exposed to 0.5 or 5 nM PLA_2 toxins and enzymes or 50 nM PLA_2 toxins for 15 min at 37° prior to TNBS labeling. Means ± SEM (N = 3–6) are shown. All of the treated values were significantly different ($P < 0.05$) from the control value. At each concentration of PLA_2, means with the letter B were significantly different from the means with the letter A ($P < 0.05$).

of PC, PE and PS (Fig. 6). When synaptosomes were treated with 0.5 nM concentrations, the greatest phospholipid hydrolysis was caused by *N. n. atra* PLA_2 (Fig. 6, top panel). In contrast, with 5 nM concentrations (Fig. 6, center panel) all four PLA_2 toxins and PLA_2 enzymes caused similar extents of phospholipid hydrolysis. Treatments with 50 nM concentrations of *N. n. atra* and *N. nigricollis* PLA_2 enzymes caused the greatest phospholipid hydrolysis, followed by notexin, with the least enzymatic activity being shown by β-BuTX (Fig. 6, bottom panel). There was no phospholipid hydrolysis in cerebrocortical SPM, even at high concentrations of PLA_2 toxins or enzymes (5 or 50 nM) when Ca^{2+} was removed and 10 mM EDTA added to the incubation solution (N = 3; data not shown). This is consistent with the known Ca^{2+} dependence of phospholipases A_2 [59].

DISCUSSION

Both the PLA_2 toxins (β-BuTX, notexin) and PLA_2 enzymes (*N. nigricollis, N. n. atra*) cause phospholipid hydrolysis and alterations in synaptosomal ACh release although the PLA_2 toxins have lower hydrolytic activity, greater lethality and act, unlike the PLA_2 enzymes, specifically at presynaptic sites [14, 15]. It was of interest to study the effects of these proteins on aminophospholipid asymmetry of SPM because: (1) a strong interaction exists between the aminophospholipids, synaptic vesicles and the cytoskeletal proteins (including synapsin) found in the brain [28, 29, 31, 32, 60, 61]; phosphorylation of synapsin is involved in the process of transmitter release from the presynaptic nerve terminals in the brain [62]; (2) PLA_2 toxins and PLA_2 enzymes alter neurotransmitter release from brain synaptosomes [for a review see Ref. 15]; (3) PLA_2 toxins and PLA_2 enzymes inhibit phosphorylation of cytoskeletal proteins in the isolated synaptosomes [40]; and (4) alteration of aminophospholipid asymmetry in the SPM has been correlated with changes in dopamine release from synaptosomes [39]. In our aminophospholipid asymmetry studies, we used TNBS to covalently label the exposed amino groups of PE and PS. TNBS has been chosen as the aminophospholipid labeling

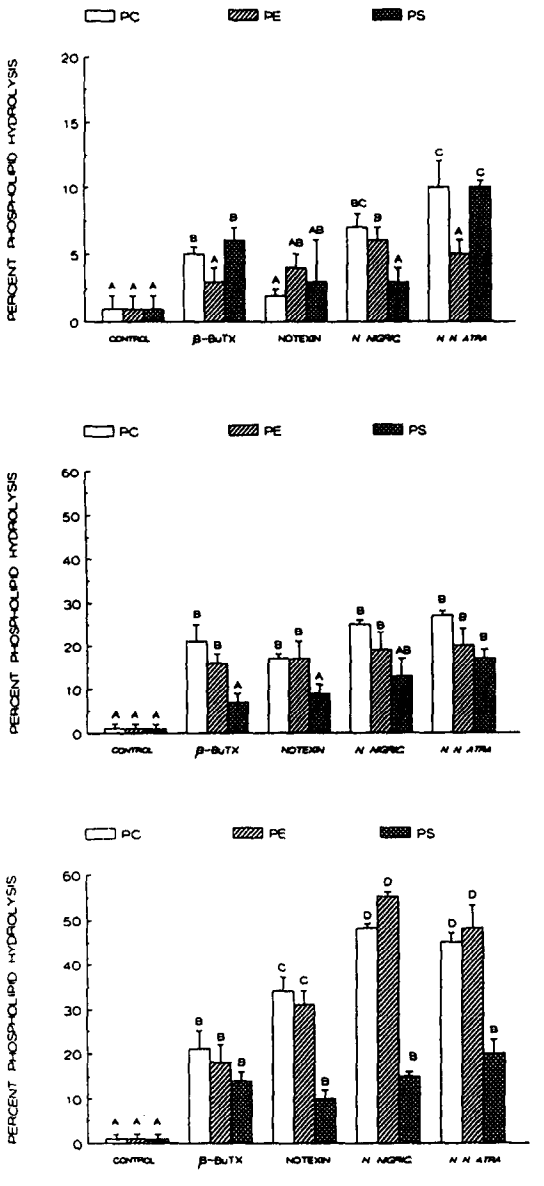

Fig. 6. Effects of PLA_2 toxins and PLA_2 enzymes on phospholipid hydrolysis in rat cerebrocortical SPM. Top panel: 0.5 nM; center panel, 5 nM; and bottom panel, 50 nM. Note that the ordinate in the center and bottom portions of the figure is different from that in the top portion. All results are presented as means ± SEM ($N > 3$). In the top (0.5 nM), means with the letter B, BC or C were significantly different from the controls (letter A). In the center and bottom (5 and 50 nM), all treated values were significantly different ($P < 0.05$) from the corresponding control values. For each phospholipid, letters are used to compare the different treatments to each other. For each phospholipid, means with different letters were significantly different ($P < 0.05$). The percent distribution of phospholipids in the control SPM were as follows ($N = 7$): PC = 45 ± 1; PE = 36 ± 2; PS = 9 ± 2; sphingomyelin = 5 ± 0.7; and phosphatidylinositol = 2 ± 0.4. This distribution was not affected significantly by any of the concentrations of the PLA_2 toxins and PLA_2 enzymes shown in this figure. The control total lipid phosphorus value in SPM was 66 ± 3 μg phosphorus/mg protein and also was not affected by the PLA_2 toxins or PLA_2 enzymes.

probe using a number of different membrane systems including red blood cell membrane [63, 64], sarcoplasmic reticulum [65], synaptosomes [24, 42], brain microsomes [66], Golgi membranes of hepatocytes [67] and synaptic vesicles [68]. The only major perturbation of the membrane that TNBS causes under impermeable conditions is the formation of a covalent bond between itself and the reactive amino groups of aminophospholipids [51].

In order for our aminophospholipid asymmetry studies to be reliable, the following conditions must be met: (1) the SPM preparations should be relatively pure and free of mitochondrial contamination; (2) TNBS must label only the accessible pools of PE and PS in the outer leaflet of the SPM without disrupting synaptosomal membrane integrity; (3) there must be sufficient TNBS to label all of the aminophospholipids on the outer leaflet of the plasma membrane; (4) the rate of flip-flop of phospholipids across the lipid bilayer must be slow compared to the duration of the labeling experiment; and (5) the PLA_2 toxins and PLA_2 enzymes must not cause disruption of synaptosomal integrity. Enrichment and relative purity of our synaptosomal and SPM preparations were determined by electron microscopy and enzyme marker assays (see Materials and Methods; results not shown). Although it is well known that these preparations are not 100% pure, they are the best available synaptic membrane models for asymmetry studies. The purity and enrichment of our synaptosomal and SPM preparations were similar to those previously used for asymmetry studies [24]. We found the appropriate concentration and incubation time for TNBS to be 2 mM and 40 min, respectively. At 2 mM TNBS, a plateau in labeling of PE (30 ± 2%) was observed without labeling of any PS. Since, in permeabilized synaptosomes, under similar conditions of TNBS concentration and time of incubation, nearly all of the PE and PS reacts with TNBS, we conclude that TNBS labeled all of the accessible pool of PE in the outer leaflet of intact synaptosomes without disrupting synaptosomal membrane integrity. We found that all of the PS and the majority of the PE were located in the inner leaflet of the SPM. This asymmetrical arrangement of aminophospholipids is similar to that found in red blood cells, platelets and fibroblasts [51, 63, 69–71]. The inaccessible pools of PE and PS were 16 and 18%, respectively, which are in agreement with the results of Fontaine *et al.* [24] where equal percentages (20%) of PE and PS were inaccessible to TNBS. In agreement with the requirements of condition (4), we found that the "flip-flop" or transbilayer movement of PE or PS was insignificant during the TNBS labeling period. If there was significant transbilayer movement of PE and PS, a continuous increase in labeling with time of these two phospholipids rather than a plateau in labeling would have been observed. However, we found that no PS was labeled with 2 mM TNBS while a plateau in labeling of PE (~30%) was reached at 30 min and extended over the next 90 min (Fig. 2). The increased percentage of PS and PE in the outer leaflet of the SPM following exposure to the PLA_2 toxins and enzymes appears to be due to

redistribution from the inner to the outer leaflet of the SPM.

The PLA_2 enzymes caused significant increases in the percentage of PE on the outer leaflet of the SPM at a 5 nM concentration while a 50 nM concentration of the PLA_2 toxins was required for a similar effect (Fig. 3). The effects of the PLA_2 toxins and PLA_2 enzymes were more marked on PS asymmetry than on PE asymmetry with all concentrations tested causing large increases in the percentage of PS labeled in the SPM (Fig. 4). These increases of PE and PS in the outer leaflets of the SPM could either be due to an increased permeability to TNBS following the exposure of the synaptosomes to the PLA_2 toxins and PLA_2 enzymes or due to a redistribution of PE and PS from the inner to the outer leaflets of the SPM. An increased permeability of the synaptosomes to TNBS is contradicted by the lack of synaptosomal membrane disruption, following a 15-min exposure to 0.5 or 5 nM PLA_2 toxins and PLA_2 enzymes or 50 nM β-BuTX, as determined by measurement of [^{3}H]*d*-glucose-6-phosphate release [21; unpublished results]. Glucose-6-phosphate is an appropriate marker to measure synaptosomal membrane disruption since it has a similar molecular weight as TNBS and, thus, a lack of effect on its release suggests that TNBS permeability is also not affected. Leakage of lactate dehydrogenase from synaptosomes was also not affected by these treatments (unpublished results). However, these non-disruptive concentrations caused significant increases in the percentage of PE and PS in the outer leaflet of the SPM (Figs. 3 and 4).

We did not measure the effects of the PLA_2 toxins and PLA_2 enzymes on the total accessible PE and PS (inner plus outer pools) in the lysed synaptosomes. Therefore, it is not known whether they unmasked the PE or PS that was in the inaccessible pools. Even so, this cannot be the only explanation for the effects of the PLA_2 toxins and PLA_2 enzymes on PS asymmetry, since the inaccessible pool of PS was 18% while at a 5 nM concentration they caused at least a 30% increase in PS labeling by TNBS (Fig. 4). Thus, the most likely explanation for our results is that a redistribution of PE and PS occurred from the inner to the outer leaflet of the SPM. The rapid redistribution of aminophospholipids caused by agents that perturb membrane structure has also been observed in red blood cells exposed to chlorpromazine, perazine and primaquine [72].

Due to the large substrate-dependent variation in reported enzymatic activity when using PLA_2 assays [19–21, 73], we measured phospholipid hydrolysis directly in the SPM after exposure of the synaptosomes to 0.5, 5 and 50 nM concentrations of the PLA_2 toxins and PLA_2 enzymes. We had found previously, using *in vitro* PLA_2 assays and artificial substrates (PC micelles and egg yolk), the following enzymatic potencies: *N. n. atra* PLA_2 > *N. nigricollis* PLA_2 > β-BuTX = notexin [21]. In contrast, when we measured phospholipid hydrolysis in the synaptosomes (Fig. 6), we found markedly different results. At 0.5 and 5 nM concentrations, there was no marked distinction between the potencies of the PLA_2 toxins and PLA_2 enzymes in inducing phospholipid hydrolysis. Even at a 50 nM concentration, the difference in potencies of the PLA_2 toxins and PLA_2 enzymes in inducing PC or PE hydrolysis in synaptosomes (2-fold) was not as great as that reported using artificial substrates [19–21]. Therefore, our findings confirm that in order to determine whether a correlation exists between enzymatic activity and membranal or functional effects, of PLA_2 toxins and PLA_2 enzymes, in synaptosomes, it is essential to measure phospholipid hydrolysis in the SPM rather than by relying on the results of PLA_2 assays using artificial substrates.

The phospholipid hydrolysis induced by the PLA_2 toxins and PLA_2 enzymes in the cerebrocortical SPM was prevented by the removal of Ca^{2+} and the addition of EDTA, confirming that Ca^{2+} is essential for PLA_2 enzymatic activity [57]. Even in the absence of phospholipid hydrolysis in the SPM (produced by removal of extracellular Ca^{2+} and addition of EDTA), 5 nM concentrations of the PLA_2 toxins and *N. nigricollis* PLA_2 caused similar increases in PS labeling as was observed in the presence of phospholipid hydrolysis (Fig. 5). These observations, together with the fact that the asymmetry of PS, a relatively poor substrate for PLA_2, was altered to a greater extent than that of PE, a better substrate for PLA_2, suggest that there is no correlation between phospholipid hydrolysis and alterations in aminophospholipid asymmetry. This conclusion is also supported by noting the concentrations of the PLA_2 toxins and PLA_2 enzymes which induced phospholipid hydrolysis and the concentrations which altered aminophospholipid asymmetry. At 0.5 and 5.0 nM concentrations, all of the PLA_2 toxins and PLA_2 enzymes caused significant increases of PS in the outer leaflet, while only *N. n. atra* PLA_2 caused significant PS hydrolysis at both of these concentrations. At 0.5 nM concentrations, the PLA_2 toxins and PLA_2 enzymes had no effects on PE asymmetry; at this concentration β-BuTX and the PLA_2 enzymes caused significant hydrolysis of PC while *N. nigricollis* PLA_2 caused significant hydrolysis of PE, and *N. n. atra* PLA_2 and β-BuTX caused significant hydrolysis of PS. Furthermore, at a 5 nM concentration, all of the PLA_2 toxins and PLA_2 enzymes showed similar potencies in hydrolyzing PC and PE while only the PLA_2 enzymes increased the percentage of PE exposed in the SPM. These observations also suggest that the increased PS and PE in the outer leaflet of SPM is not dependent upon phospholipid hydrolysis induced by the PLA_2 toxins and PLA_2 enzymes.

Aminophospholipid asymmetry is thought to arise from: (1) protein–lipid interactions, such as the interaction between cytoskeletal proteins and aminophospholipids [29, 31, 32, 37, 74–78]. It is especially interesting that the interaction of synapsin I (the cytoskeletal protein most directly associated with neurotransmitter release [62]) with mixed phospholipid bilayers is dependent upon the presence of acidic phospholipids such as PS [27]; and (2) an ATP-dependent translocation of aminophospholipids to the inner leaflet of the membrane [25, 33, 35, 77]. The mechanism by which the PLA_2 toxins and PLA_2 enzymes caused the alterations in aminophospholipid asymmetry which we have observed is not known but may involve (1) the

disruption of the interaction between cytoskeletal proteins and aminophospholipids, and/or (2) inhibition of the ATP-dependent aminophospholipid translocase activity. The former (1) mechanism is supported by the observations that: (i) a 30-min incubation with 15 nM PLA_2 toxins and enzymes inhibits phosphorylation of synapsin I, a major cytoskeletal protein in the synaptosomes [40], hence indicating that they might have disruptive effects on the function of the cytoskeletal proteins; (ii) a 15-min exposure to a 0.5 nM concentration of the PLA_2 toxins and PLA_2 enzymes altered PS but not PE asymmetry, in agreement with the greater binding of strongly acidic phospholipids, such as PS, to synapsin [27]. The latter (2) mechanism is supported by the observations that β-BuTX uncouples oxidative phosphorylation in synaptosomal mitochondria and inhibits ATP synthesis in the brain [79]. By reduction of the energy stores in the synaptosomes, β-BuTX might inhibit the activity of the ATP-dependent aminophospholipid translocase, leading to the inability of the aminophospholipids to be translocated into the inner leaflet of the SPM. However, we have found recently that concentrations of 5 and 15 nM β-BuTx and 5 nM *N. n. atra* PLA_2 had no effect on ATP levels in synaptosomes from rat cerebral cortex (Ueno E and Rosenberg P, unpublished observations). A third possible mechanism whereby PS asymmetry could be altered by the toxins and enzymes would be a stimulation of the enzyme phosphatidylethanolamine:serine transferase which converts PE to PS. If this were to occur in the outer leaflet of the plasma membrane, it could explain our results. However, if this occurred, we would expect to see a relative increase in the percentage of PS and a decrease in the percentage of PE relative to the other phospholipids. In fact, neither the percent distribution nor the total lipid phosphorus value of SPM was affected by 0.5, 5 or 50 nM concentrations of the toxins and enzymes used in this study (see legend to Fig. 6).

This alteration in PE and PS orientation induced by the PLA_2 toxins and enzymes which we found could be of a great enough magnitude to alter membrane-mediated biological processes. Membrane-mediated processes are indeed altered in red blood cells [38] and dopamine release is altered in synaptosomes [39] after equivalent or less changes in aminophospholipid orientation in the plasma membrane. It has also been suggested that transbilayer movement of PS could be a general controlling factor in the cell biology of membrane fusion [80]. Changes in PS asymmetry in the plasma membrane, such as we have observed, or in the membrane of the synaptic vesicle might modify the ability of the acetylcholine-containing synaptic vesicles to fuse with the plasma membrane and release their neurotransmitter. While the PLA_2 toxins act specifically on cholinergic terminals in the periphery [1, 3–7], they affect the release of many different neurotransmitters (e.g. glutamine, aspartate, γ-aminobutyric acid, norepinephrine) in the central nervous system [79, 81–83], even though the specificity of some of these effects has been questioned [84]. It is therefore, not necessarily surprising that there is a large change in PS asymmetry even though synaptosomes from rat cerebral cortex are not purely or even primarily cholinergic.

Both PLA_2 toxins and PLA_2 enzymes increase acetylcholine release from resting synaptosomes [11–13, 78, 84–86] and decrease release from 4-aminopyridine-stimulated synaptosomes [84]. While the specific PLA_2 toxins and PLA_2 enzymes used in this study all alter ACh release, the toxins act in a lower concentration than the enzymes [84; Ghassemi A and Rosenberg P, unpublished observations]. In contrast, the PLA_2 enzymes were as potent or more potent than the PLA_2 toxins in altering aminophospholipid asymmetry. Thus, the changes in aminophospholipid asymmetry may not be responsible for the presynaptic specificity and potency of the PLA_2 toxins, although these changes in asymmetry may be associated with alterations in neurotransmitter release. Alternatively, a possible explanation for the differences in potencies and specificities of the PLA_2 toxins and enzymes may be that the PLA_2 enzymes non-specifically alter membranal aminophospholipid asymmetry while the PLA_2 toxins alter asymmetry much more selectively, since they have specific presynaptic binding sites [for review see Ref. 15].

Acknowledgements—This research was supported by a National Institute of Health research grant (RO1NS14521) awarded to P. R. We extend our gratitude to Dr. C-C. Yang (Institute of Life Sciences, National Tsing Hua University, Hsinchu, Taiwan, R.O.C.) for the isolation and purification of the PLA_2 toxins and enzymes and Drs. Alan Wachtel and Lamia Khairallah (Department of Molecular and Cell Biology, The University of Connecticut, Storrs, CT 06269, U.S.A.) for their assistance in the preparation and interpretation of electron micrographs. We also thank Drs. Eleanora Condrea (Department of Biochemistry, Tel-Aviv University, Israel) and Richard Crain (Department of Molecular and Cell Biology, The University of Connecticut, Storrs) for advice during the course of these studies and for review of this manuscript.

REFERENCES

1. Chang CC and Lee CY, Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action. *Arch Int Pharmacodyn Ther* **144**: 241–257, 1963.
2. Karlsson E, Eaker D and Ryden L, Purification of a presynaptic neurotoxin from the venom of the Australian tiger snake *Notechis scutatus scutatus*. *Toxicon* **10**: 405–413, 1972.
3. Abe T, Limbrick AR and Miledi R, Acute muscle denervation by β-bungarotoxin. *Proc R Soc Lond* **194**: 545–553, 1976.
4. Abe T, Alema S and Miledi R, Isolation and characterization of presynaptically acting neurotoxins from the venom of *Bungarus* snakes. *Eur J Biochem* **80**: 1–12, 1977.
5. Chang CC, Chen TF and Lee CY, Studies of the presynaptic effect of β-bungarotoxin on neuromuscular transmission. *J Pharmacol Exp Ther* **184**: 339–345, 1973.
6. Kelly RB and Brown FR, Biochemical and physiological properties of a purified snake venom neurotoxin which acts presynaptically. *J Neurobiol* **5**: 135–150, 1974.
7. Oberg SG and Kelly RB, The mechanism of β-bungarotoxin action. I. Modification of transmitter

release at the neuromuscular junction. *J Neurobiol* **7**: 129–141, 1976.
8. Gulya K, Budai D, Kása P and Rakonczay Z, *In vivo* effects of β-bungarotoxin on the acetylcholine system in different brain areas of the rat. *J Neurochem* **43**: 112–119, 1984.
9. Ng RH and Howard BD, Deenergization of nerve terminals by β-bungarotoxin. *Biochemistry* **17**: 4978–4986, 1978.
10. Rugolo M, Dolly JO and Nicholls DG, The mechanism of action of β-bungarotoxin at the presynaptic plasma membrane. *Biochem J* **233**: 519–523, 1986.
11. Sen I and Cooper JR, The effect of β-bungarotoxin on the release of acetylcholine from brain synaptosomal preparations. *Biochem Pharmacol* **24**: 2107–2116, 1975.
12. Sen I, Grantham PA and Cooper JR, Mechanism of action of β-bungarotoxin on synaptosomal preparations. *Proc Natl Acad Sci USA* **73**: 2664–2668, 1976.
13. Sen I, Baba A, Schulz RA and Cooper JR, Mechanism of action of notexin and notechis II-5 on synaptosomes. *J Neurochem* **31**: 969–976, 1978.
14. Rosenberg P, The relationship between enzymatic activity and pharmacological properties of phospholipases in natural poisons. In: *Natural Toxins: Animal, Plant, and Microbial* (Ed. Harris JB), pp. 129–174. Clarendon Press, Oxford, 1986.
15. Rosenberg P, Phospholipases. In: *Handbook of Toxinology* (Eds. Shier WT and Mebs D), pp. 68–277. Marcel Dekker, New York, 1990.
16. Yang CC and King K, Chemical modification of the histidine residues in basic phospholipase A_2 from the venom of *Naja nigricollis*. *Biochim Biophys Acta* **614**: 373–388, 1980.
17. Chang CC, Chuang S-T, Lee CY and Wei JW, Role of cardiotoxin and phospholipase A in the blockade of nerve conduction and depolarization of skeletal muscle induced by cobra venom. *Br J Pharmacol* **44**: 752–764, 1972.
18. Fletcher JE, Rapuano BE, Condrea E, Ryan M and Rosenberg P, Comparison of relatively toxic phospholipase A_2 from *Naja nigricollis* snake venom with that of a relatively non-toxic phospholipase A_2 from *Hemachatus haemachatus* snake venom. II. Pharmacological properties in relationship to enzymatic activities. *Biochem Pharmacol* **29**: 1565–1574, 1980.
19. Ho CL and Lee CY, Mode of neuromuscular blocking action of ceruleotoxin. *Toxicon* **21**: 301–307, 1983.
20. Lee CY and Ho CL, The pharmacology of phospholipase A_2 isolated from snake venoms, with particular reference to their effects of neuromuscular transmission. In: *Advances in Pharmacology and Therapeutics II* (Eds. Yoshida H, Hagihara Y and Ebashi S), Vol. 4, pp. 37–52. Pergamon Press, New York, 1982.
21. Yates SL, Burns M, Condrea E, Ghassemi A, Shina R and Rosenberg P, Phospholipid hydrolysis and loss of membrane integrity following treatment of rat brain synaptosomes with β-bungarotoxin, notexin, and *Naja naja atra* and *Naja nigricollis* phospholipase A_2. *Toxicon* **28**: 929–951, 1990.
22. Bishop WR and Bell RM, Assembly of phospholipids into cellular membranes: Biosynthesis, transmembrane movement and intracellular translocation. *Annu Rev Cell Biol* **4**: 579–610, 1988.
23. Butler M and Morrell P, Sidedness of phospholipid synthesis in brain membranes. *J Neurochem* **39**: 155–164, 1982.
24. Fontaine R, Harris R and Schroeder F, Aminophospholipid asymmetry in murine synaptosomal plasma membrane. *J Neurochem* **34**: 269–277, 1980.
25. Zachowski A and Devaux PF, Transmembrane movements of lipids. *Experientia* **46**: 644–656, 1990.
26. Zwaal RFA, Membrane and lipid involvement in blood coagulation. *Biochim Biophys Acta* **515**: 163–205, 1978.
27. Benfenati F, Greengard P, Brunner J and Bähler M, Electrostatic and hydrophobic interactions of synapsin I and synapsin I fragments with phospholipid bilayers. *J Cell Biol* **108**: 1851–1862, 1989.
28. Comfurius P, Bevers EM and Zwaal RFA, Interaction between phosphatidylserine and the isolated cytoskeleton of human blood platelets. *Biochim Biophys Acta* **983**: 212–216, 1989.
29. Franck PFH, Op den Kamp JAF, Roelofsen B and van Deenen LLM, Does diamide treatment of intact human erythrocytes cause a loss of phospholipid asymmetry? *Biochim Biophys Acta* **857**: 127–130, 1986.
30. Haest CWM, Interactions between membrane skeleton protein and the intrinsic domain of erythrocyte membrane. *Biochim Biophys Acta* **694**: 331–352, 1985.
31. Maksymiw R, Sui SF, Gaub H and Sackmann E, Electrostatic coupling of spectrin dimers to phosphatidylserine containing lipid lamellae. *Biochemistry* **26**: 2983–2990, 1987.
32. Rybicki AC, Heath R, Lubin B and Schwartz RS, Human erythrocyte protein 4.1 is a phosphatidylserine binding protein. *J Clin Invest* **81**: 255–260, 1988.
33. Zwaal RFA, Bevers EM, Comfurius P, Rosing J, Tilly RHJ and Verhallen PJF, Loss of membrane phospholipid asymmetry during activation of blood platelets and sickled cells; Mechanisms and physiological significance. *Mol Cell Biochem* **91**: 23–31, 1989.
34. Connor J and Schroit AJ, Transbilayer movement of phosphatidylserine in nonhuman erythrocytes: Evidence that the aminophospholipid transporter is a ubiquitous membrane protein. *Biochemistry* **28**: 9680–9685, 1989.
35. Devaux PF, Static and dynamic lipid asymmetry in cell membranes. *Biochemistry* **30**: 1163–1173, 1991.
36. Schroit AJ, Madsen JW and Tanaka Y, *In vivo* recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. *J Biol Chem* **260**: 5131–5138, 1985.
37. Franck PFH, Bevers EM, Lubin BH, Comfurius P, Chiu DTY, Op den Kamp JAF and Zwaal RFA, Uncoupling of the membrane cytoskeleton from the lipid bilayer. *J Clin Invest* **75**: 183–190, 1985.
38. Jain SK, The accumulation of malonaldehyde, a product of fatty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes. *J Biol Chem* **259**: 3391–3394, 1984.
39. Bradford P and Marinetti GV, Effects of local anesthetics on phospholipid topology and dopamine release in rat brain synaptosomes. *J Membr Biol* **67**: 211–218, 1982.
40. Ueno E and Rosenberg P, Inhibition of phosphorylation of rat synaptosomal proteins by snake venom phospholipase A_2 neurotoxins (β-bungarotoxin, notexin) and enzymes (*Naja naja atra, Naja nigricollis*). *Toxicon* **28**: 1423–1437, 1990.
41. Bradford P and Marinetti GV, Trinitrobenzenesulfonic acid and fluorodinitrobenzene: Probes to study local anesthetic effects in cell membranes. *J Membr Biol* **67**: 193–198, 1981.
42. Fontaine RN, Harris A and Schroeder F, Neuronal membrane lipid asymmetry. *Life Sci* **24**: 395–400, 1979.
43. Means GE, Congdon WI and Bender ML, Reactions of 2,4,6-trinitrobenzenesulfonate ion with amines and hydroxide ion. *Biochemistry* **11**: 3564–3571, 1972.
44. Yang CC, King K and Sun TP, Chemical modification of lysine and histidine residues in phospholipase A_2 from the venom of *Naja naja atra* (Taiwan cobra). *Toxicon* **19**: 645–660, 1981.
45. Yang CC and Chang LS, Tryptophan modification of phospholipase A_2 enzymes and presynaptic neurotoxins from snake venoms. *J Protein Chem* **3**: 195–213, 1984.

46. Booth RFG and Clark JB, A rapid method for preparation of relatively pure metabolically active synaptosomes from rat brain. *Biochem J* **176**: 365–370, 1978.
47. Cottman CW and Matthews DA, Synaptic plasma membranes from rat brain synaptosomes: Isolation and partial characterization. *Biochim Biophys Acta* **249**: 380–394, 1971.
48. Rubin MS and Tzagoloff A, Cytochrome oxidase of *Saccharomyses cerevisae*. *Methods Enzymol* **53**: 73–79, 1978.
49. Johnson MK, The intracellular distribution fo glycolytic and other enzymes in rat brain homogenate and mitochondrial preparations. *Biochem J* **77**: 610–618, 1960.
50. Bonting SL, Simon KA and Hawkins NM, Studies on sodium-potassium-activated adenosine triphosphatase. I. Quantitative distribution in several tissues of the cat. *Arch Biochem Biophys* **95**: 416–423, 1961.
51. Gordesky S, Marinetti G and Love R, The reaction of chemical probes with the erythrocyte membrane. *J Membr Biol* **20**: 111–132, 1975.
52. Crain RC and Marinetti GV, Phospholipid topology of the inner mitochondrial membrane of rat liver. *Biochemistry* **18**: 2407–2414, 1979.
53. Jiang M, Haggblad J, Heilbronn E, Rydqvist B and Eaker D, Biochemical characteristics and cell-mediated action of a toxic phospholipase A_2 (AgTX) isolated from the venom of the pit viper (*Agkistrodon halys*, Pallas). *Toxicon* **25**: 785–792, 1987.
54. Walum E and Peterson A, Tritiated 2-deoxy-D-glucose as a probe for cell membrane permeability studies. *Anal Biochem* **120**: 8–11, 1981.
55. Marinetti GV, Albrecht M, Ford T and Stotz E, Analysis of human plasma phosphatides by paper chromatography. *Biochim Biophys Acta* **36**: 4–13, 1959.
56. Folch J, Lees J and Sloane-Stanley GH, A simple method for the isolation and purification of total lipids from animal tissue. *J Biol Chem* **226**: 497–509, 1957.
57. Condrea E, Rosenberg P and Dettbarn WD, Determination of phospholipid splitting as the factor responsible for increased permeability and block of axonal conduction induced by snake venom. I. Study on lobster axons. *Biochim Biophys Acta* **135**: 669–681, 1967.
58. Bartlett GR, Phosphorus assay in column chromatography. *J Biol Chem* **234**: 466–468, 1959.
59. Verheij HM, Slotboom AJ and de Haas GH, Structure and function of phospholipase A_2. *Rev Physiol Biochem Pharmacol* **91**: 92–203, 1981.
60. De Camilli P, Harris SM Jr, Huttner WB and Greengard P, Synapsin I (protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J Cell Biol* **96**: 1355–1373, 1983.
61. Huttner WB, Schiebler W, Greengard P and De Camilli P, Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J Cell Biol* **96**: 1374–1388, 1983.
62. Hemmings HG, Angus AC, McGuinness TL, Huganir RL and Greengard P, Role of protein phosphorylation in neuronal signal transduction. *FASEB J* **3**: 1583–1592, 1989.
63. Gordesky S and Marinetti GV, The asymmetric arrangement of phospholipids in the human erythrocyte membrane. *Biochem Biophys Res Commun* **50**: 1027–1031, 1973.
64. Kahlenberg A, Walker C and Rohrlick R, Evidence for an asymmetric distribution of phospholipids in the human erythrocyte membrane. *Can J Biochem* **52**: 803–806, 1974.
65. Vale MGP, Localization of aminophospholipids in sarcoplasmic reticulum membranes revealed by trinitrobenzosulfonate and fluorodinitrobenzene. *Biochim Biophys Acta* **471**: 39–48, 1977.
66. Erhardt A, Leray C, Binaglia L, Roberti R, Dreyfus H, Massarelli R and Freysz L, *In vitro* synthesis and transbilayer movement of phosphatidylethanolamine molecules labeled with different fatty acids in chick brain microsomes. *Biochim Biophys Acta* **1021**: 126–132, 1990.
67. Higgins JA, The transverse distribution of phospholipids in the membranes of Golgi subfractions of rat hepatocytes. *Biochem J* **219**: 261–272, 1984.
68. Deutch JW and Kelly RB, Lipids of synaptic vesicles: Relevance to the mechanism of membrane fusion. *Biochemistry* **20**: 378–385, 1981.
69. Marinetti GV and Crain RC, Topology of aminophospholipids in the red cell membranes. *J Supramol Struct* **8**: 191–213, 1978.
70. Schick AJ, Madsen J and Chacko GK, Radioiodinated, photoactivatable phosphatidylethanolamine and phosphatidylserine: Transfer properties and differential photoreactive interaction with human erythrocyte membrane proteins. *Biochemistry* **26**: 1812–1819, 1987.
71. Fontaine RN and Schroeder F, Plasma membrane aminophospholipid distribution in transformed murine fibroblasts. *Biochim Biophys Acta* **558**: 1–12, 1979.
72. Tamura A, Moriwaki N and Fujji T, Disturbing effect of cationic amphiphilic drugs on phospholipid asymmetry of the membrane lipid bilayer of human erythrocytes. *Chem Pharm Bull (Tokyo)* **13**: 1692–1704, 1983.
73. Strong PN, Goerke J, Oberg SG and Kelly RB, β-Bungarotoxin, a pre-synaptic toxin with enzymatic activity. *Proc Natl Acad Sci USA* **73**: 178–182, 1976.
74. Dressler V, Haest CWM, Plasa G, Deuticke B and Erusalimsky JD, Stabilizing factors of phospholipid asymmetry in the erythrocyte membrane. *Biochim Biophys Acta* **775**: 189–196, 1984.
75. Geiger B, Membrane–cytoskeleton interactions. *Biochim Biophys Acta* **737**: 305–341, 1983.
76. Marinetti GV and Love R, Differential reaction of cell membrane phospholipids and proteins with chemical probes. *Chem Phys Lipids* **16**: 239–254, 1976.
77. Middelkoop E, Lubin BH, Bevers EM, Op den Kamp JAF, Comfurius P, Chiu DT, Zwaal RFA, van Deenen LLM and Roelofsen B, Studies on sickled erythrocytes provide evidence that the asymmetric distribution of phosphatidylserine in the red cell membrane is maintained by both ATP-dependent translocation and interaction with membrane skeletal proteins. *Biochim Biophys Acta* **937**: 281–288, 1988.
78. Venien C and Le Grimellec C, Phospholipid asymmetry in renal brush-border membranes. *Biochim Biophys Acta* **942**: 159–168, 1988.
79. Wernicke JF, Vanker AD and Howard BD, The mechanism of action of β-bungarotoxin. *J Neurochem* **25**: 483–496, 1975.
80. Baldwin JM, O'Reilly R, Whitney M and Lucy JA, Surface exposure of phosphatidylserine is associated with the swelling and osmotically-induced fusion of human erythrocytes in the presence of Ca^{2+}. *Biochim Biophys Acta* **1028**: 14–20, 1990.
81. Halliwell JV, Tse CK, Othman I and Dolly JO, Biochemical and electrophysiological demonstrations of the actions of β-bungarotoxin on synapses in brain. *J Neurochem* **39**: 543–550, 1982.
82. Smith CCT, Bradford HF, Thompson EJ and MacDermot J, Actions of β-bungarotoxin on amino acid transmitter release. *J Neurochem* **34**: 487–494, 1980.
83. Wernicke JF, Oberjat T and Howard BD, β-Neurotoxin

reduces neurotransmitter storage in brain synapses. *J Neurochem* **22**: 781–788, 1974.
84. Chapell R and Rosenberg P, Specificity and action of β-bungarotoxin on acetylcholine release from synaptosomes. *Toxicon* **30**: 621–633, 1992.
85. Fletcher JE and Middlebrook JL, Effects of beta-bungarotoxin and *Naja naja atra* snake venom phospholipase A_2 on acetylcholine release and choline uptake in synaptosomes. *Toxicon* **24**: 91–99, 1985.
86. Nicholls D, Snelling R and Dolly O, Bioenergetic actions of β-bungarotoxin, dendrotoxin, and bee-venom phospholipase A_2 on guinea-pig synaptosomes. *Biochem J* **229**: 653–662, 1985.