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ANTIBODIES HAVING MARKEDLY DIFFERENT EFFECTS ON ENZYMATIC ACTIVITY AND INDUCTION OF ACETYLCHOLINE RELEASE BY TWO PRESYNAPTICALLY-ACTING PHOSPHOLIPASE A_2 NEUROTOXINS

JEFFREY E. FLETCHER,*†‡ MING-SHI JIANG* and JOHN L. MIDDLEBROOK§

Departments of *Anesthesiology and †Biochemistry, Hahnemann University, Philadelphia, PA 19102–1192; and §Toxinology Division, USAMRIID, Frederick, MD 21702–5011, U.S.A.

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Abstract—The enzymatic and acetylcholine-releasing activities of two presynaptically-acting phospholipase A_2 neurotoxins (pseudexin B and scutoxin) were studied in a synaptosomal fraction. Scutoxin (100 nM) induced greater [^{14}C]acetylcholine release than did pseudexin B (100 nM). Both toxins caused fatty acid production in the synaptosomal fraction, although pseudexin B was more active than scutoxin. One monoclonal antibody raised against pseudexin B (#4) had no effect on the enzymatic activity of either pseudexin B or scutoxin. Two other monoclonal antibodies (#3 and #7), also raised against pseudexin B, antagonized the enzymatic activity of pseudexin B and scutoxin. Monoclonal antibody #3 was more effective than #7 in reducing the amount of acetylcholine released by the toxins, whereas #7 was more effective than #3 in reducing fatty acid production. Although antibody #3 caused complete inhibition of phospholipase A_2 activity of pseudexin B on purified substrates, it only reduced phospholipase A_2 activity by 35% in synaptosomes. These findings support the hypothesis that gross phospholipase A_2 activity does not play a role in stimulation of acetylcholine release by the presynaptically-acting phospholipase A_2 neurotoxins.

Key words: pseudexin B; scutoxin; snake venom; neurotransmitter release; phospholipid; fatty acid

Among the components of some snake venoms are very potent presynaptically-acting toxins that initially stimulate, then block the release of ACh‖ at the neuromuscular junction [1–4]. A common feature of these neurotoxins is the presence of PLA_2 (EC 3.1.1.4) activity. The PLA_2 enzymes catalyze the hydrolysis of fatty esters at the #2 position of phospholipids, liberating primarily unsaturated fatty acids. However, the role of PLA_2 activity in the neurotoxicity of these toxins is controversial [4–12]. These toxins stimulate ACh release from synaptosomal fractions [13–17]. However, some venom PLA_2 enzymes that are inactive at the neuromuscular junction are also potent stimulators of ACh release from synaptosomal fractions [13, 15, 18], although they may be generally less potent than the PLA_2 with a presynaptic action at the neuromuscular junction [19].

The fatty acids produced by PLA_2 activity could account, in part, for the toxicity of these enzymes, as fatty acids alter the functions of several membrane-bound proteins [20–23], including potassium channels [9]. Most studies examining the role of enzymatic activity in the action of presynaptically-acting PLA_2 toxins have determined the enzymatic activity on purified substrates and extrapolated these findings to the biological systems used for studying neuromuscular transmission. A number of years ago, Rosenberg [24] cautioned against over-interpretation of the results from such studies. Subsequently, studies have demonstrated that PLA_2 activity occurs in biological systems under conditions in which it is abolished using purified substrate systems [25, 26], further emphasizing the need to examine enzyme activity and toxicity in the same preparation.

The present study was designed to examine the effects of pseudexin B and scutoxin on ACh release from synaptosomes and to analyze fatty acid production by the neurotoxins in mammalian synaptosomes, using gas chromatography. The effects of three pseudexin directed monoclonal antibodies were studied with these presynaptically-acting PLA_2 neurotoxins to determine if stimulation of ACh release can be accounted for by their PLA_2 activity. The PLA_2 activities were also determined on the same synaptosomal substrate used for the studies of ACh release, making extrapolation of data from other systems unnecessary. Fatty acid-free BSA binds fatty acids [27]. Thus, at least some of the potentially toxic fatty acids generated by PLA_2 activity can be removed from the synaptosomal membrane into the incubation medium containing BSA. BSA was used in the synaptosomal preparations, as it preferentially antagonizes the toxic actions of PLA_2 that are inactive at the neuromuscular

‡ Corresponding author: Jeffrey E. Fletcher, Ph.D., Department of Anesthesiology MS-310, Hahnemann University, Philadelphia, PA 19102–1192. Tel. (215) 762–3506; FAX (215) 762–8656.

‖ Abbreviations: ACh, acetylcholine; and PLA_2, phospholipase A_2.

junction, relative to the actions of presynaptically-acting PLA_2 [28, 29]. Thus, by reducing nonspecific fatty acids associated with the synaptosomes while retaining toxicity, BSA allows us to better elucidate the role of specific fatty acids remaining in the synaptosomal membrane in stimulation of ACh release by the presynaptically-acting PLA_2.

MATERIALS AND METHODS

Materials. Scutoxin was obtained from Ventoxin Laboratories, Inc. (Melbourne, FL). Pseudexin B was prepared as previously described [30]. The production and characterization of antibodies to pseudexin, including cross-reactivity with other toxins, were also described previously [31]. The numbers designating the antibodies in the publication in which they were initially characterized [31] were retained in the present study for cross-reference. Lipid standards, HEPES, Percoll, ACh, neostigmine and fatty acid-free BSA were purchased from the Sigma Chemical Co. (St. Louis, MO). [^{14}C]Choline (50–60 mCi/mmol) was obtained from Amersham (Arlington Heights, IL).

Synaptosome preparation. An enriched synaptosomal fraction was prepared from mouse brain [32]. Briefly, whole brains from three Swiss Webster (Harlan Sprague–Dawley, Indianapolis, IN) mice (20–25 g) were homogenized (3 g tissue/9 mL) in sucrose (0.32 M), EDTA (1 mM), dithiothreitol (0.25 mM), pH 7.4 (4°). The homogenate was centrifuged (1000 *g*; 10 min), and the supernatant volume was adjusted to 13 mL. Aliquots of the supernatant (4 mg protein/mL) were applied to a discontinuous Percoll gradient (2 mL each of 23%, 15%, 10% and 3% Percoll; v/v; pH 7.4) and centrifuged at 32,500 *g* for 15 min. The interfaces between 10 and 15% and 15 and 23% Percoll [32] were pooled for use in the ACh release and fatty acid analyses.

[^{14}C]*Acetylcholine release.* [^{14}C]ACh was quantitated by a tetraphenylboron and 3-heptanone extraction technique and liquid scintillation counting, as previously described [15]. Synaptosomes were preloaded with [^{14}C]choline (2 μM) for 30 min (25°) to synthesize [^{14}C]ACh in Incubation Buffer comprised of (in mM): HEPES, 10; NaCl, 137; KCl, 2.7; $CaCl_2$, 1.7; $MgCl_2$, 0.7; D-glucose, 20; and adjusted to pH 7.4 with 1 N HCl. Unincorporated [^{14}C]choline was removed by three washes (microcentrifuge; 30 sec) with Incubation Buffer containing neostigmine (100 μM). Antibodies were preincubated with the toxin at a ratio of 80:1 (antibody:toxin; w/w) for 30 min at 25° in Incubation Buffer. Aliquots of synaptosomes (0.5 mg each) in 0.5 mL of Incubation Buffer containing neostigmine (100 μM) and BSA (0.5%) were incubated with or without toxin and antibody at 25° for 30 min. The synaptosomes were centrifuged (Eppendorf 5414 microcentrifuge; 30 sec) and the supernatants transferred to a separate tube. A solution containing choline kinase was added to eliminate choline and to selectively extract ACh into tetraphenylboron and 3-heptanone [15]. An aliquot of the organic phase was then quantitated by liquid scintillation counting, using Scinti Verse™II (Fisher Scientific Co., Pittsburgh, PA).

Gas chromatographic fatty acid analysis. Synaptosomes were prepared and incubated with or without toxin and antibody for 30 min with BSA (0.5%) present in the buffer, as described above. The synaptosomal incubates (0.5 mg protein/0.5 mL) were centrifuged (Eppendorf 5414 microcentrifuge; 30 sec) and the lipids were extracted separately from the supernatant (containing BSA-bound fatty acids) and pellets (containing the fatty acids resistant to extraction from the synaptosomes by BSA) with $CH_3OH:CHCl_3$ (3:1) by the methods of Marinetti *et al.* [33] and Folch *et al.* [34], as previously described [22, 35]. The neutral lipids were separated by one-dimensional thin-layer chromatography, the free fatty acids methylated [36] and the fatty acid methyl esters separated on a Shimadzu (Columbia, MD) GC-9A gas chromatograph and quantitated using Beckman (Allendale, NJ) System Gold software, as previously described [22, 35]. Heptadecanoic acid was added to the extract as an internal standard [22, 35]. Adding all of the individual fatty acids (each peak in the gas chromatogram) would yield separate total values for the supernatant or pellet. These separately determined pellet and supernatant values could then be added together to yield the total PLA_2 activity for either the individual fatty acids, or the total fatty acids. All values are expressed as picomoles per milligram of protein. Protein was determined by a modification [37] of the method of Lowry *et al.* [38]. The mean control values (see Fig. 2 legend) were subtracted from the toxin-treated preparations to yield the fatty acid produced by the enzymatic activity presented in Figs. 2–4.

Enzymatic activity on purified substrate. Pseudexin B was preincubated with or without the indicated amount of antibody #3 at 25° for 60 min in Tris buffer containing Tris (100 mM), $CaCl_2$ (2 mM) and BSA (0.5%), adjusted to pH 7.4 with 1 N HCl. The toxin (0.3 μg) was then added to 1 mL Tris buffer containing mixed micelles of Triton X-100 (2 mM) and egg yolk phosphatidylcholine (1 mM) and incubated at 37° for 30 min [39]. Free fatty acids were extracted and titrated by the method of Dole [40].

Statistics. For the ACh release studies, each experiment was conducted in triplicate, and data from 2–5 different experiments (N = 6–15; see Fig. 1) were pooled and the results expressed as means ± SD. A one-way analysis of variance ($P < 0.05$) and Scheffe test were conducted separately for each of the toxins to examine the effects of the antibodies on ACh release. Gas chromatographic analysis of fatty acids was conducted in duplicate in one experiment and as a single determination in a second experiment and, since the results were similar, the data were pooled together and the results expressed as means ± SD (N = 3). Since antibodies #3, #4 and #7 were demonstrated previously to inhibit or have no effect on PLA_2 activity on purified substrates [31], a one-tailed *t*-test ($P < 0.05$) was used to determine the inhibition by each antibody treatment in relation to its respective control. Other statistical analyses are specifically indicated in Results.

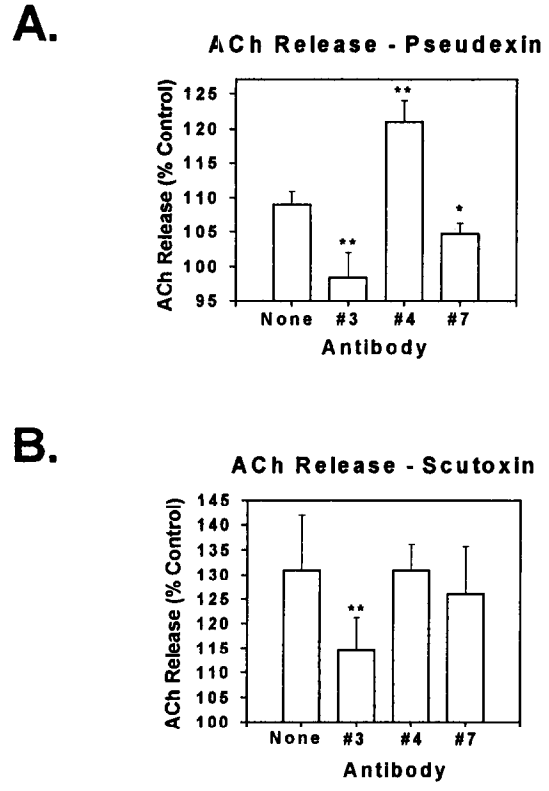

Fig. 1. Effects of antibodies #3, #4 and #7 on ACh release by presynaptically-acting PLA_2 neurotoxins. Pseudexin B (100 nM)- and scutoxin (100 nM)-induced [^{14}C]ACh release from synaptosomes incubated for 30 min at 25° was determined in the presence of BSA (0.5%). The results are expressed as the means ± SD for the percent of spontaneous [^{14}C]ACh release from control preparations. The numbers of determinations for pseudexin B and scutoxin in the absence of antibodies were 12 and 15, respectively. The values for pseudexin and scutoxin in the absence of antibodies (None) were significantly different ($P < 0.0001$); two-tailed paired *t*-test) from controls with no toxin. The numbers of determinations in the presence of antibodies for both toxins were nine for #3, and six each for #4 and #7. In panel A, the values significantly different from pseudexin B alone are indicated by * ($P < 0.025$) and ** ($P < 0.0001$). In panel B, the values significantly different from scutoxin alone are indicated by ** ($P < 0.002$). See Materials and Methods for statistical analysis.

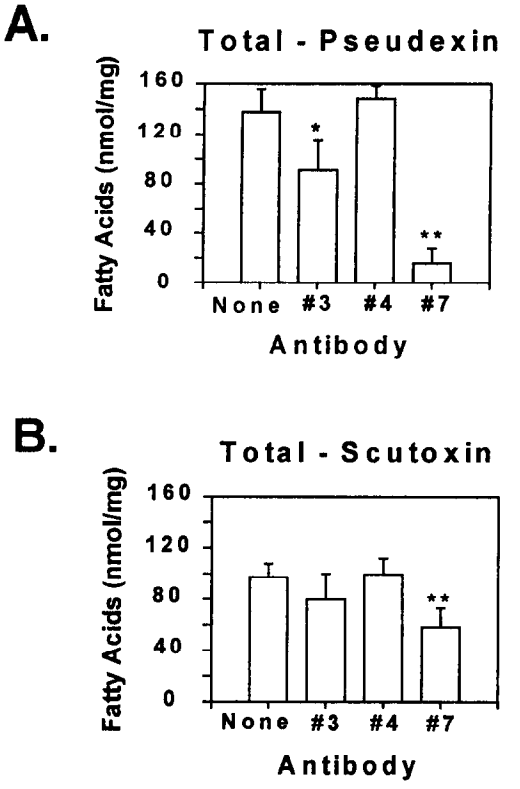

Fig. 2. Total fatty acid production by presynaptically-acting PLA_2 neurotoxins. Pseudexin (100 nM; panel A) and scutoxin (100 nM; panel B) were incubated with synaptosomes in the presence of BSA (0.5%) for 30 min at 25°. Values are the means ± SD for three determinations. In panel A, the values significantly different from pseudexin B alone are indicated by * ($P < 0.03$) and ** ($P < 0.003$). In panel B, the values significantly different from scutoxin alone are indicated by ** ($P < 0.01$). The free fatty acid composition of control supernatants and synaptosomal pellets, respectively, were (means ± SD in pmol/mg): total (includes some minor fatty acids) 9000 ± 510 and 14,000 ± 340; palmitic (16:0) 2000 ± 140 and 2500 ± 800; stearic (18:0) 1100 ± 150 and 2200 ± 390; oleic (18:1) 1600 ± 110 and 2300 ± 180; arachidonic (20:4) 1400 ± 150 and 2000 ± 460; and docosahexanoic (22:6) 2800 ± 400 and 4900 ± 1300. These mean values were subtracted from the toxin-treated preparations to yield the fatty acid produced by the enzymatic activity in Figs. 2–4.

RESULTS

[^{14}C]*Acetylcholine release.* Under our experimental conditions, scutoxin stimulated ACh release from synaptosomes to a significantly ($P < 0.0001$; two-tailed grouped *t*-test) greater extent than did pseudexin B (Fig. 1). Neither of these toxins released greater than 20% of the total releasable ACh. Two additional PLA_2 with no known presynaptic action at the neuromuscular junction, one from *Naja naja atra* and one from *Naja naja kaouthia* venom (both at 100 nM), did not elicit ACh release with BSA included in the incubation medium (data not shown).

We examined the effects of several monoclonal antibodies previously reported to influence the enzymatic and biological activities of pseudexin [31] on toxin-stimulated ACh release. Antibodies #3 and #7 antagonized the stimulatory action of pseudexin B on ACh release (Fig. 1A). Antibody #3 was significantly ($P < 0.002$) more effective than #7 and completely blocked the action of pseudexin B (Fig. 1A). In contrast, monoclonal antibody #4 increased the pseudexin B-induced ACh release (Fig. 1A). Although all three monoclonal antibodies exhibited indistinguishable reactions with notexin and scutoxin by ELISA (data not shown), only #3 had any significant action on scutoxin-stimulated ACh release from synaptosomes (Fig. 1B).

PLA_2 activity. In addition to exerting many

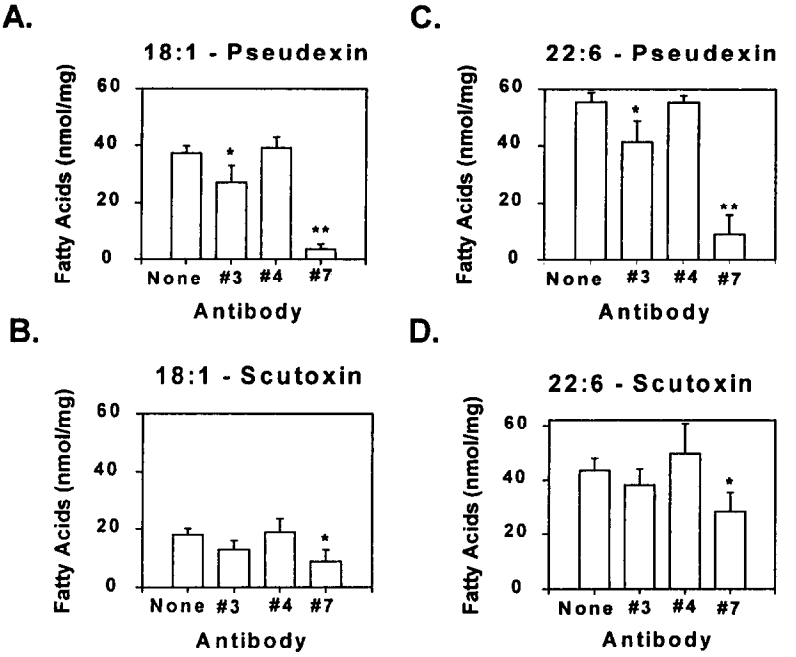

Fig. 3. The 18:1 and 22:6 fatty acid production by presynaptically-acting PLA_2 neurotoxins. Pseudexin (100 nM; panels A and C) and scutoxin (100 nM; panels B and D) were incubated with synaptosomes in the presence of BSA (0.5%) for 30 min at 25°. Values are the means ± SD for three determinations. In panel A, the values significantly different from pseudexin B alone are indicated by * ($P < 0.03$) and ** ($P < 0.0001$). In panels B, C and D the values significantly different from pseudexin or scutoxin alone are indicated by * ($P < 0.02$), or ** ($P < 0.0002$).

biological effects, PLA_2 toxins are enzymes releasing fatty acids from synthetic and natural sources. We analyzed the types and amounts of fatty acids produced by the action of pseudexin and scutoxin on synaptosomal phospholipids. Since synaptosomes have a basal level of free fatty acids, the mean values in the absence of toxin (see Fig. 2 legend) were subtracted from the toxin-treated preparations to yield the fatty acid produced by toxin-associated PLA_2 activity in Figs. 2–4.

The total (pellet plus supernatant) free fatty acid produced in the presence of BSA by pseudexin B (Fig. 2) exceeded that produced by scutoxin ($P < 0.03$; two-tailed grouped *t*-test). Comparing the four major fatty acids released (Figs. 3 and 4), the relative proportions of 16:0 (20%) and 20:4 (16%) were similar for both enzymes. Likewise, the percentage of 18:1 released by pseudexin B (26%) and scutoxin (19%) were close, as were the percentage of 22:6 released by scutoxin (45%) and pseudexin B (38%). Therefore, the compositions of the acyl groups esterified to the phospholipid substrates attacked by the two enzymes were similar.

Since the synaptosomal pellet was extracted separately from the incubation medium, it was possible to analyze the extraction of fatty acids by BSA. The fatty acids extracted by BSA accounted for about 15% of the total fatty acid production of pseudexin B and about 25% of that of scutoxin in the absence of antibodies (data not shown). Therefore, the bulk of fatty acids produced by treatment with either toxin remains in the synaptosomes. The antibodies had less of an effect on the total fatty acids extracted by BSA than on those retained in the synaptosomes. For example, with pseudexin B, antibody #7 caused total fatty acids extracted by BSA to decrease from 20 ± 3.2 (mean ± SD) to 6.6 ± 5.6 nmol/mg whereas total fatty acids retained in the pellet (not extracted by BSA) decreased from 118 ± 18 to 9.3 ± 6.3 nmol/mg. With scutoxin, antibody #7 caused total fatty acids extracted by BSA to decrease from 26 ± 6.9 to 20 ± 4.0 nmol/mg and fatty acids retained in the pellet to decrease from 72 ± 8.3 to 38 ± 12 nmol/mg.

Both pseudexin B and scutoxin exhibited a preference for substrates containing 22:6 at the #2 position (Fig. 3). The least preferred fatty acid appeared to be 20:4 (Fig. 4), with 18:1 (Fig. 3) and 16:0 (Fig. 4) in between. Overall, the effects of the antibodies on liberation of each of the individual fatty acids by pseudexin and scutoxin were similar. That is, antibody #7 inhibited all types of fatty acids liberated by both toxins and to a greater extent than did antibody #3 (Figs. 3 and 4). However, while antibody #3 inhibited all types of fatty acids liberated by pseudexin, 20:4 was the only fatty acid liberated by scutoxin that was inhibited significantly by this antibody (Figs. 3 and 4).

Since antibody #3 is reported to inhibit enzymatic activity of pseudexin B on purified substrates [31], but was relatively ineffective in inhibiting PLA_2

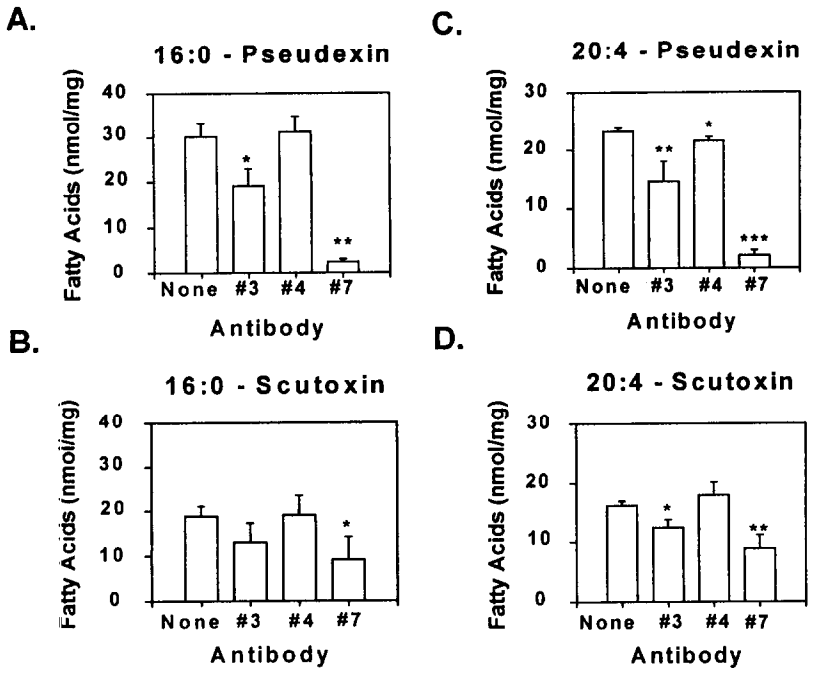

Fig. 4. The 16:0 and 20:4 fatty acid production by presynaptically-acting PLA_2 neurotoxins. Pseudexin (100 nM; panels A and C) and scutoxin (100 nM; panels B and D) were incubated with synaptosomes in the presence of BSA (0.5%) for 30 min at 25°. Values are the means ± SD for three determinations. In panel A, the values significantly different from pseudexin B alone are indicated by * ($P < 0.01$) and ** ($P < 0.0001$). In panel B, the values significantly different from scutoxin alone are indicated by * ($P < 0.02$). In panel C, the values significantly different from pseudexin B alone are indicated by * ($P < 0.02$), ** ($P < 0.01$) and *** ($P < 0.0001$). In panel D, the values significantly different from scutoxin alone are indicated by * ($P < 0.01$) and ** ($P < 0.004$).

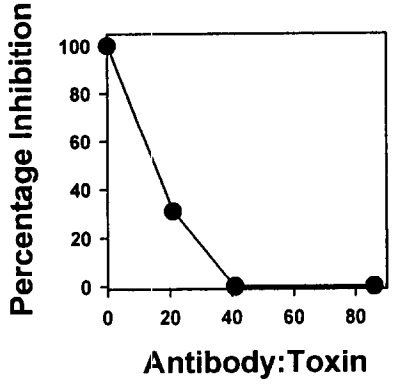

Fig. 5. Effects of antibody #3 on the PLA_2 activity of pseudexin B on artificial substrates. The effects on PLA_2 activity were determined on Triton X-100:phosphatidylcholine mixed micelle substrates incubated with toxin for 30 min at 37°. The values are the means of duplicate determinations. The two control values for fatty acid release were 33 and 35 μmol/mg·min.

activity in synaptosomes (Figs. 2–4), we examined the concentration–response inhibition of enzymatic activity on purified substrates by this antibody. Antibody #3 completely antagonized the PLA_2 activity of pseudexin B on purified substrates, at half of the concentration used in the synaptosomal studies (Fig. 5). Thus, monoclonal antibody #3 was used in the synaptosomal studies at a ratio to toxin that should have been very effective in inhibiting fatty acid release; however, the efficacy of this antibody in inhibiting PLA_2 activity is, apparently, greatly decreased on biological substrates.

DISCUSSION

Pseudexin B and scutoxin were shown to stimulate ACh release in synaptosomal preparations, in agreement with their presynaptic action in the phrenic nerve-diaphragm preparation [41]. Scutoxin and notexin are isolated from the same venom (*Notechis scutatus scutatus*) and differ by only two amino acids.* Antibodies #3, 4 and 7 all bind equally to pseudexin B and notexin, as judged by ELISA [31]. The effects of these antibodies on pseudexin B and scutoxin were similar in that #3 was the most

* Kaiser II, personal communication. Cited with permission.

effective in antagonizing ACh release and #7 was the most effective in antagonizing PLA_2 activity for both toxins. On the other hand, the effects of these antibodies on ACh release did not necessarily correlate with the effects on enzyme activity. For example, fatty acid production by pseudexin B was decreased 90% by antibody #7, whereas ACh release was only decreased by 50%. More striking was the complete blockade of ACh release by antibody #3, but only a 35% decrease in fatty acid production. Also, the determination of PLA_2 activity on purified substrates could not be extrapolated to the synaptosomal preparation, as demonstrated with antibody #3.

Pseudexin B has an LD_{50} value of about 1000 μg/kg [31], whereas scutoxin has an LD_{50} value of about 6 μg/kg [42]. Therefore, these toxins differ by more than 150-fold in their lethal potency, but they did not differ markedly (*ca.* 3-fold) in their effects on ACh release in the synaptosomal preparation. We presently do not understand this anomaly. Also, pseudexin B has a greater enzymatic activity on synaptosomal preparations than scutoxin. Therefore, even in the absence of antibodies there is a better correlation between lethality and ACh release than lethality and enzymatic activity.

Of 15 monoclonal antibodies examined, only #3 and #7 neutralized the lethal effects of pseudexin B [31]. While none of these antibodies neutralized the lethal effects of notexin, antibody #7, which was the most effective in inhibiting ACh release from synaptosomes, consistently prolonged the time to death following injection of this toxin [31]. The neutralizing action of antibody #7 would be in agreement with our findings regarding inhibition of scutoxin-induced ACh release.

The fact that each of the antibodies bound similarly to pseudexin B and scutoxin, yet produced different degrees of change in the biological activities we measured may seem contradictory. However, even with only two amino acid substitutions, it is possible that the native conformations of pseudexin B and scutoxin have folded differently. Moreover, we were unable to determine whether the inhibitory or stimulatory responses described were due to simple steric effects of binding, or to antibody-induced conformational alterations of the antigens. In the latter case, it could be that antibody binding to a conserved structural region of two proteins could produce different effects in other conserved (e.g. catalytic) regions of the molecules.

No other studies have examined the action of presynaptically-acting PLA_2 neurotoxins on ACh release from synaptosomes in the presence of BSA. However, one group of investigators has examined the effects of BSA on membrane disruption caused by the *Naja naja atra* PLA_2, notexin and β-bungarotoxin [28] and observed almost complete inhibition of the *Naja naja atra* PLA_2-induced membrane disruption by BSA. Yates *et al.* [28] also reported that only fatty acids, not lysophospholipids, were removed from synaptosomes by BSA.

The present study was the first to examine the fatty acid production of the presynaptically-acting PLA_2 toxins in mammalian synaptosomal preparations. Regarding the liberation of specific fatty acids by these presynaptic PLA_2 toxins, substrates containing 22:6 were the most readily attacked. The #2 position of phospholipids is believed to be primarily occupied by unsaturated fatty acids. However, significant amounts of 16:0 were released by these PLA_2 enzymes, in agreement with the presence of approximately 25% of dipalmitoyl phosphatidylcholine in rat brain [43, 44]. The levels of 18:0 or other minor fatty acids (16:1; 18:2; 18:3) were not increased significantly by the PLA_2 (data not shown). Thus, although relatively abundant, 18:0 does not occupy the #2 position of phospholipids to any significant extent in synaptosomes. This pattern has been reported for red blood cells [35, 45, 46]. The relatively low levels of hydrolysis of 18:2 in synaptosomes (data not shown) contrasts with the extensive hydrolysis of this fatty ester by PLA_2 in red blood cells [35, 45, 46], but is consistent with the virtual absence of this fatty ester on phospholipids in the brain [43, 44, 47–49]. There appeared to be much more 22:6 relative to 20:4 in the synaptosomes than in skeletal muscle [50].

[^{14}C]ACh is primarily a reflection of the release of newly synthesized pools of ACh. These stores of ACh are acted on by the toxins, as has been demonstrated previously by a number of investigators [13–17] and as shown in Fig. 1. However, it is still possible that the release of other stores of ACh may be affected differently by the toxins and antibodies.

A localized generation of small amounts of specific fatty acids could still account for the neurotoxicity of PLA_2 presynaptic neurotoxins, as even under the most extreme condition of enzyme inhibition retaining the stimulation of ACh release (pseudexin and antibody #7), there was still detectable enzyme activity. However, the present findings support previous studies suggesting that PLA_2 activity must be determined on the biological substrate used for toxicity testing and that gross fatty acid production does not play a role in the stimulation of ACh release by these toxins.

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