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Differential Effects of Snake Venom Phospholipase A_2 Neurotoxin (β-Bungarotoxin) and Enzyme (*Naja naja atra*) on Protein Kinases

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ABSTRACT. The phospholipase A_2 (PLA_2) neurotoxin, β-bungarotoxin (β-BuTX), presynaptically alters acetylcholine release. We previously found that β-BuTX inhibits protein phosphorylation in rat brain synaptosomes. This inhibition was not due to the inhibition of ATP synthesis, the action of arachidonic acid (AA) metabolites, or the stimulation of phosphatase activities. A typical PLA_2 enzyme from *Naja naja atra* (*N. n. atra*) venom also inhibited phosphorylation but with lesser potency than that of β-BuTX. We now report the effects of β-BuTX and *N. n. atra* PLA_2 on the activities of protein kinases. Treatments of synaptic plasma membrane or cytosol with *N. n. atra* PLA_2 stimulated the activities of cAMP-dependent kinase, Ca^{2+}/calmodulin-dependent kinase II, and protein kinase C (PKC), whereas β-BuTX had no effect on these kinases. Calyculin A, a phosphatase-1 and -2A inhibitor, increased the stimulation of phosphorylation by *N. n. atra* PLA_2, indicating that the stimulation is not due to an inhibition of phosphatase activities. The stimulation of PKC by *N. n. atra* PLA_2 appears to be mediated by free fatty acids (FFAs) resulting from phospholipid hydrolysis by PLA_2, since (1) treatment of either synaptic plasma membrane or cytosol with *N. n. atra* PLA_2 produced large amounts of FFAs, and (2) AA, an exogenous FFA, stimulated PKC activity to an extent similar to that caused by *N. n. atra* PLA_2. Thus, the mechanisms of action of β-BuTX and *N. n. atra* PLA_2 appear quite different from each other although both agents inhibit phosphorylation in intact synaptosomes. BIOCHEM PHARMACOL 52;8:1287–1293, 1996.

KEY WORDS. phospholipase A_2; β-bungarotoxin; protein kinase A; protein kinase C; CaM-kinase II; free fatty acids

β-BuTX,† one of the snake venom neurotoxins, possesses PLA_2 (EC 3.1.1.4) activity and presynaptically inhibits acetylcholine release at neuromuscular junctions [1]. The presynaptic action of β-BuTX has also been demonstrated in the CNS [2] where its action appears to be specific for cholinergic nerve terminals [3]. β-BuTX shows a remarkable homology in amino acid sequence to most snake venom PLA_2 enzymes such as *Naja naja atra* (*N. n. atra*) PLA_2 [4]. These snake venom PLA_2 enzymes, however, have much lower lethal potencies than the PLA_2 neurotoxins [5], and they cause less specific and less potent effects on acetylcholine release [3]. It has been suggested that the effects on neurotransmitter release by PLA_2 neurotoxins are correlated with their enzymatic activities [1, 6], while other studies indicated a dissociation between neurotoxicity and phospholipid hydrolysis [7, 8].

Phosphorylation of synaptosomal proteins such as synapsin I and GAP-43 modulates neurotransmitter release [9, 10]. Synapsin I is a synaptic vesicle-associated phosphoprotein, and its phosphorylation by CaM-kinase II increases the number of synaptic vesicles in the releasable pool within nerve terminals, resulting in an increase in neurotransmitter release [10]. PKC has also been shown to modulate neurotransmitter release [11]. GAP-43 and MARCKS, both of which are PKC substrates and mainly reside on presynaptic nerve terminal plasma membrane, bind calmodulin in a PKC-catalyzed phosphorylation-dependent manner [12, 13]. Phosphorylation of GAP-43 increases neurotransmitter release [9], and MARCKS becomes phosphorylated in response to depolarization of synaptosomes [14]. MARCKS also cross-links F-actin in a PKC-catalyzed phosphorylation-dependent manner, suggesting its possible

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† *Abbreviations:* Ac-MBP_{4-14}, acetylated myelin basic $protein_{4-14}$; AA, arachidonic acid; β-BuTX, β-bungarotoxin; CaM-kinase II, Ca^{2+}/calmodulin-dependent kinase II; FFA, free fatty acid; *N. n. atra*, *Naja naja atra*; OAG, 1-oleoyl-2-acetoyl-sn-glycerol; α-PDD, 4α-phorbol didecanoate; PKA, cAMP-dependent kinase; PKC, protein kinase C; PLA_2, phospholipase A_2; PMA, 4β-phorbol 12-myristate 13-acetate; and PS, phosphatidylserine.

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role as a PKC signal mediator [15]. An alteration in phosphorylation of these synaptosomal phosphoproteins by an action on ATP synthesis, protein kinases, phosphoprotein phosphatases, or substrates would be expected to alter neurotransmitter release.

In investigating the neurotoxic mechanism of β-BuTX, we found that β-BuTX inhibits phosphorylation of many proteins [16], most potently CaM-kinase II-dependent phosphorylation of synapsin [17] and PKC-dependent phosphorylation of GAP-43 and MARCKS [18]. *N. n. atra* PLA_2 showed a similar although weaker effect than β-BuTX on synapsin I phosphorylation. Its effects on phosphorylation of PKC substrates, however, are different from those of β-BuTX since it stimulates basal phosphorylation of these substrates similarly to phorbol esters which are PKC stimulants [18]. As part of our continuing investigation into the inhibitory mechanism of β-BuTX on protein phosphorylation, we have now demonstrated that β-BuTX does not directly affect the activities of PKC, CaM-kinase II, or PKC, whereas *N. n. atra* PLA_2 causes a stimulation of these kinases.

MATERIALS AND METHODS

Materials

β-BuTX and *N. n. atra* PLA_2 were purchased from Ventoxin Laboratories (Frederick, MD). [^{32}P]ATP (6000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). PMA and α-PDD were obtained from the Sigma Chemical Co. (St. Louis, MO). Calyculin A and KN-62 were obtained from LC Laboratories (Woburn, MA) and Calbiochem-Novabiochem International (San Diego, CA), respectively. Kemptide, Ac-MBP_{4-14}, autocamtide, PKA inhibitor peptide$_{6-22}$ amide, PKC inhibitor peptide$_{19-36}$ and negatively charged p81 phosphocellulose disk sheets were purchased from Life Technologies, Inc. (Gaithersburg, MD). An FFA assay kit was obtained from Wako Chemicals USA, Inc. (Richmond, VA). All other reagents were of analytical grade.

Isolation of Cytosol and Synaptic Plasma Membrane from Rat Brain Synaptosomes

Synaptosomes were isolated from cerebral cortices of male Sprague-Dawley rats (110–125 g) as described previously [16]. Synaptosomes were hypoosmotically lysed in 2 mM Tris-HCl (pH 7.4) containing 4-(2-aminoethyl)-benzenesulfonylfluoride (0.2 mM), aprotinin (2 μg/mL), EDTA (1 mM), leupeptin (10 μg/mL), and pepstatin A (10 μg/mL), followed by centrifugation at 100,000 *g* for 60 min. The supernatant was collected as the cytosolic fraction. The pellet was resuspended in 4.5 mL of the lysis buffer, layered on top of a discontinuous sucrose gradient (4.5 mL of 0.8 M and 4.5 mL of 1.2 M sucrose containing 1 mM Tris-HCl, pH 7.4), and centrifuged at 200,000 *g* for 30 min. The interface between 0.8 M and 1.2 M sucrose, which contained the synaptic plasma membrane, was collected, centrifuged at 15,000 *g* for 16 min, washed once, and resuspended in the lysis buffer.

Assays for PKA, CaM-kinase II, and PKC

Our kinase assays were modified from those in the literature for PKA [19], CaM-kinase II and PKC [19, 20] in addition to instruction manuals for these kinase assays provided by Life Technologies, Inc. PKA and CaM-kinase II activities were assayed by incubating synaptic plasma membrane (2 μg protein) at 30° for 5 min in 50 μL of phosphorylation buffer (40 μM [γ-^{32}P]ATP (40 μCi/mL), 10 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5). PKC activities were assayed by incubating cytosol (2 μg protein) in phosphorylation buffer (50 μL) at 30° for 30 min unless otherwise specified. The incubation medium also contained exogenous peptide substrates (50 μM kemptide for PKA, 20 μM autocamtide for CaM-kinase II, and 50 μM Ac-MBP_{4-14} for PKC) and activators [10 μM cAMP for PKA, 1 μM calmodulin plus 2.5 mM $CaCl_2$ for CaM-kinase II and multilamellar liposomes (composed of 10 μg/mL PS, 1 μg/mL OAG) plus 1 mM $CaCl_2$ for PKC]. In some experiments 0.5% (v/v) Triton X-100 micelles, composed of 280 μg/mL PS and 10 μM PMA, were used as the lipid activator for PKC assays instead of liposomes as described above. Nonspecific phosphorylation for each kinase assay was determined by the presence of 1 μM PKA inhibitor peptide$_{6-22}$ amide for PKA, 10 μM KN-62 for CaM-kinase II, and 20 μM PKC inhibitor peptide$_{19-36}$ for PKC. The nonspecific phosphorylation of PKA, CaM-kinase II, and PKC was less than 15, 12, and 3% of the total phosphorylation, respectively, and these values were subtracted. In the case of PKA and CaM-kinase II, the effects of *N. n. atra* PLA_2 and β-BuTX were determined by preincubating synaptic plasma membrane (3–4 mg protein/mL) with these agents at 30° for 30 min in 2 mM $CaCl_2$ and 50 mM Tris-HCl (pH 7.4). The preincubation reaction was stopped by adding 2.5 mM EGTA, and the pretreated synaptic plasma membrane (2 μg protein) was subjected to phosphorylation assay at 30° for 5 min as previously described. In the case of PKC, the effects of *N. n. atra* PLA_2 and β-BuTX were determined by incubating the cytosolic fraction (2 μg protein) with these agents at 30° for 30 min during the phosphorylation reaction (total volume 50 μL). AA (400 μM) in ethanol was freshly prepared by sonicating sodium-AA at 0° for 5 min under a stream of nitrogen and then was appropriately diluted with 20 mM Tris-HCl (pH 7.5). In some experiments, 100 nM calyculin A was present during the phosphorylation, and the control samples for these experiments contained 0.1% (v/v) DMSO. At the end of the incubation, an aliquot was spotted on a phosphocellulose disk, followed by extensive washing with 1% (v/v) phosphoric acid and subsequently with water, and bound radioactivities were measured by scintillation counting.

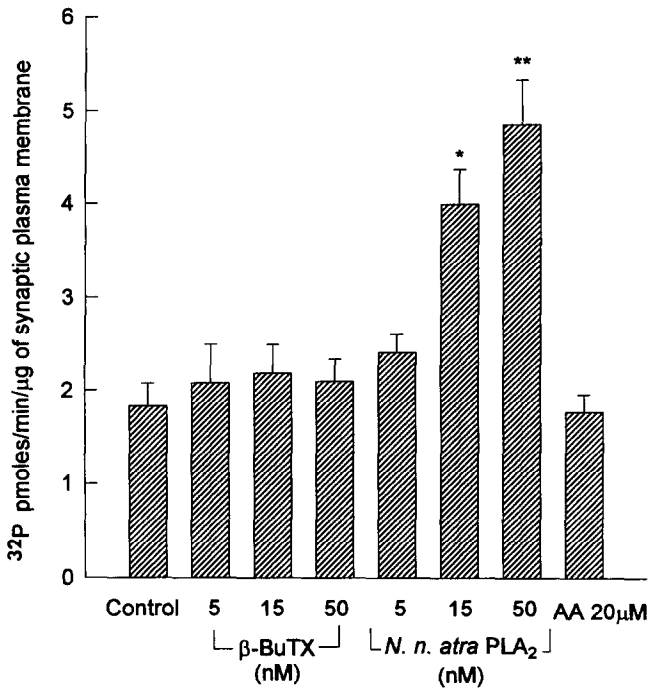

FIG. 1. Effects of *N. n. atra* PLA_2 and β-BuTX on PKA activity in synaptic plasma membrane. Synaptic plasma membrane (3–4 mg protein/mL) was preincubated at 30° for 30 min in 2 mM $CaCl_2$ and 50 mM Tris-HCl (pH 7.4), in the absence (control) or the presence of *N. n. atra* PLA_2 and β-BuTX at 5, 15, and 50 nM. Preincubation was stopped by adding 2.5 mM EGTA. Pretreated synaptic plasma membrane (2 μg protein) was subjected to PKA assay (total volume 50 μL) as described in Materials and Methods. The effect of AA was determined by adding AA to the preincubated controls during the phosphorylation assay. Data are shown as the means ± SEM from three separate experiments. Key: (*) significantly different from control ($P < 0.0001$); and (**) significantly different from control and from (*) ($P < 0.0001$).

Measurement of FFAs in Cytosol of Synaptic Plasma Membrane

Synaptic plasma membrane (1.5 mg protein/mL) or cytosol (0.5 mg protein/mL) in incubation buffer (3 mM $CaCl_2$, 0.9 mM EDTA, and 50 mM Tris-HCl, pH 7.4) was pretreated with either β-BuTX or *N. n. atra* PLA_2 (1, 5, and 50 nM) at 30° for 30 min. Incubation was terminated by the addition of EGTA (3 mM final concentration). The pretreated synaptic plasma membrane (40 μg protein) or cytosol (13 μg protein) was subjected to FFA measurement according to the method provided by the manufacturer. Briefly, added CoA is acylated by FFAs in the presence of added acyl-CoA synthetase. The acyl-CoA thus produced is oxidized by added acyl-CoA oxidase with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase, permits the oxidative condensation of 3-methyl-*N*-ethyl-*N*-(β-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple-colored adduct that can be measured at 550 nm. Oleic acid which was contained in an assay kit was used as a standard, and the data provided by the manufacturer showed that AA can be measured with an activity of 89% and a recovery of 85% relative to that of oleic acid.

Statistical Analysis

Statistical significance was determined using an analysis of variance followed by Duncan's multiple range test. The statistical analysis was performed under the release 82.2B of the SAS Institute (Cary, NC).

RESULTS

The effects of β-BuTX and *N. n. atra* PLA_2 on protein kinases, in cytosol and synaptic plasma membrane, were determined in *in vitro* assays using endogenous kinases and exogenous peptide substrates. In controls, specific activities of PKA, CaM-kinase II, and PKC (pmol of ^{32}P incorporated into peptide substrate/min/μg protein) in cytosol and synaptic plasma membrane fractions showed a linearity within 1–5 μg protein per assay. In investigating the distribution of these kinase activities, it was found that the specific activity of PKA was similar in synaptic plasma membrane and cytosol. Specific activity of CaM-kinase II was 6- to 8-fold

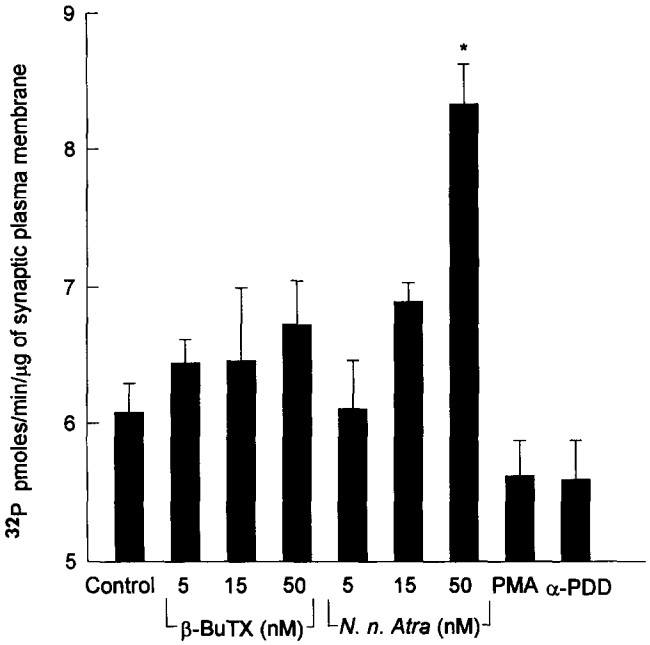

FIG. 2. Effects of *N. n. atra* PLA_2 and β-BuTX on CaM-kinase II activity in synaptic plasma membrane. Synaptic plasma membrane was pretreated with *N. n. atra* PLA_2 and β-BuTX at 5, 15, and 50 nM at 30° for 30 min as described in the legend of Fig. 1. The pretreated synaptic plasma membrane (2 μg protein) was subjected to a phosphorylation assay at 30° for 5 min as described in Materials and Methods. Effects of PMA and α-PDD were determined by adding these agents to the preincubated controls during the phosphorylation assay. Data are shown as the means ± SEM from three separate experiments. Key: (*) significantly different from control and from other treatments ($P < 0.0003$).

higher in synaptic plasma membrane than in cytosol, whereas, in contrast, PKC activity was 5- to 10-fold higher in cytosol than in synaptic plasma membrane. Based on these preliminary studies, either synaptic plasma membrane or cytosol was used for determining the effects of *N. n. atra* PLA_2 and β-BuTX on PKA, CaM-kinase II, or PKC activities.

N. n. atra PLA_2 at 15 and 50 nM concentration dependently stimulated PKA activity in synaptic plasma membrane (Fig. 1). AA, one of FFAs resulting from phospholipid hydrolysis by PLA_2, did not have any significant effect even at 20 μM which is a higher concentration than the estimated concentration of FFAs generated by *N. n. atra* PLA_2 (described below). β-BuTX (5, 15, and 50 nM) had no effect on PKA activity (Fig. 1). *N. n. atra* PLA_2 at 50 nM also stimulated CaM-kinase II activity in synaptic plasma membrane, whereas, in contrast, β-BuTX did not have any effect (Fig. 2). Also, both PMA and α-PDD, active and inactive phorbol esters, respectively, had no effect on CaM-kinase II activity (Fig. 2). *N. n. atra* PLA_2 (5, 15, and 50 nM) significantly stimulated PKC activity in cytosol using PS/OAG liposomes as the lipid activator (Fig. 3). There were no differences between these different concentrations of *N. n. atra* PLA_2 in stimulating PKC (Fig. 3). The stimulation of PKC by *N. n. atra* PLA_2 was significantly greater when calyculin A, a potent inhibitor for phosphatase-1 and -2A, was present during the phosphorylation reaction (Fig. 4). *N. n. atra* PLA_2 did not have any stimulatory effect on PKC activity when Triton X-100 micelles, composed of PS and PMA, were used as the lipid activator (data not shown). β-BuTX, in contrast to *N. n. atra* PLA_2 did not have any effect on PKC activity even at the high concentration of 50 nM (Fig. 3). Exogenous AA (5–20 μM), in the presence of PS/OAG, stimulated PKC activity to an extent similar to that caused by *N. n. atra* PLA_2 (Fig. 5). As seen with *N. n. atra* PLA_2, AA did not cause any significant stimulation of PKC activity when Triton X-100 micelles, composed of PS and PMA, were used as the lipid activator (Fig. 5).

Figure 6 shows FFA production by treatments of cytosol and synaptic plasma membrane with *N. n. atra* PLA_2 and β-BuTX. In synaptic plasma membrane, production of FFAs by β-BuTX was less than that of *N. n. atra* PLA_2. In cytosol, β-BuTX produced significant amounts of FFAs at 50 nM; however, the quantity was less than that of 5 nM *N. n. atra* PLA_2. Concentrations of 5 and 50 nM *N. n. atra* PLA_2 were equipotent in producing FFAs in the cytosolic fraction which contains markedly fewer phospholipids than are present in synaptic plasma membrane. *N. n. atra* PLA_2 at 5 and 50 nM produced 122 ± 3 and 153 ± 27 nmol FFAs/mg of cytosolic protein (Fig. 6), respectively, which gave rise, under the conditions of our phosphorylation assay, to final FFA concentrations of 4.9 and 6.1 μM, while β-BuTX at 50 nM produced 67 ± 5 nmol of FFAs/mg of cytosolic protein (Fig. 6), which gave rise to a final concentration of 2.7 μM.

DISCUSSION

In previous studies, we found that a potent PLA_2 neurotoxin, β-BuTX, inhibits the phosphorylation of many syn-

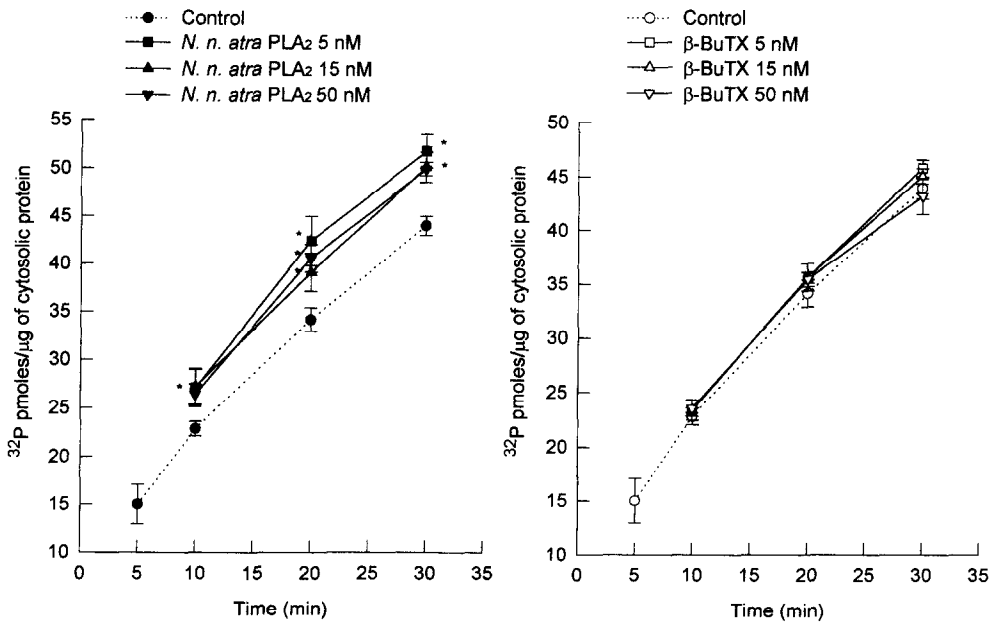

FIG. 3. Effects of *N. n. atra* PLA_2 and β-BuTX on PKC activity in cytosolic fraction. The activity of PKC was measured by incubating cytosol (2 μg protein) in phosphorylation buffer (50 μL) at 37° for various times either in the absence (control) or presence of *N. n. atra* PLA_2 or β-BuTX. Incubation was terminated by spotting an aliquot of the reaction mixture on a phosphocellulose disk. Data are shown as the means ± SEM from three separate experiments at each time point. Key: *significantly different from control ($P < 0.02$).

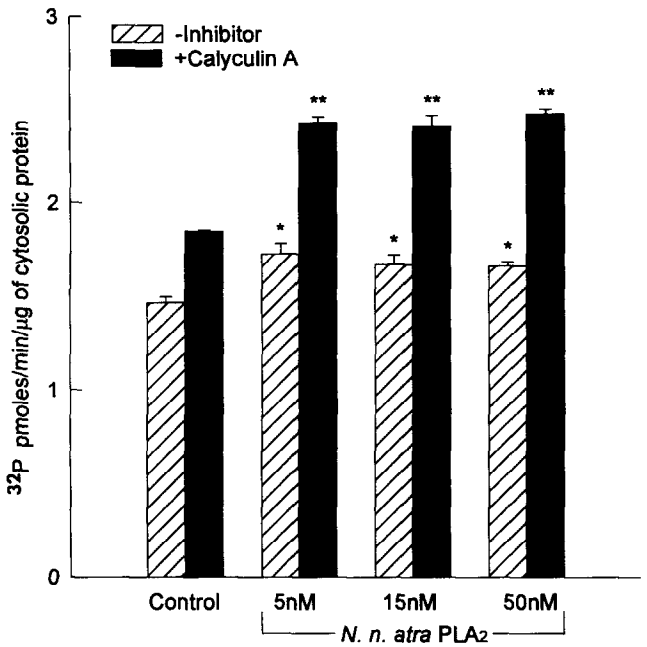

FIG. 4. Effects of *N. n. atra* PLA_2 on cytosolic PKC activity in the presence of the phosphatase inhibitor calyculin A. Cytosolic fraction (2 μg protein) was incubated in phosphorylation buffer (50 μL) at 30° for 30 min in the absence or presence of *N. n. atra* PLA_2. Hatched columns indicate phosphorylation in the absence of calyculin A but in the presence of 0.1% (v/v) DMSO as vehicle control. There was no significant effect of DMSO on phosphorylation. Closed columns indicate phosphorylation in the presence of 100 nM calyculin A. Data are shown as the means ± SEM from three separate experiments. Key: (*) significantly different from control in the absence of calyculin A ($P < 0.0005$); and (**) significantly different from control in the presence of calyculin A and from *N. n. atra* PLA_2 in the absence of calyculin A ($P < 0.0001$).

aptosomal proteins [16], including CaM-kinase II-dependent phosphorylation of synapsin I [17] and PKC-dependent phosphorylation of GAP-43 and MARCKS [18]. In these studies, the effects on phosphorylation of *N. n. atra* PLA_2, a typical PLA_2 enzyme that does not have a specific action on neurotransmitter release, were compared with those of β-BuTX to determine whether the enzymatic activity of β-BuTX was essential for its pharmacological action. *N. n. atra* PLA_2 also inhibits (but to a lesser extent) phosphorylation of synapsin I [17]; however, in contrast to β-BuTX, it decreases synaptosomal ATP levels by 63% [17] and also stimulates phosphorylation of PKC substrates similar to that observed with a PKC stimulant, PMA [18].

In the present study, we found that β-BuTX had no direct effects on the activities of PKA, CaM-kinase II, or PKC (Figs. 1–3) at concentrations (5, 15, and 50 nM) that caused potent inhibition of phosphorylation of synapsin I, GAP-43, and MARCKS [16–18]. In contrast, *N. n. atra* PLA_2 stimulated these kinase activities although the extent of stimulation and the effective concentration varied in the different kinase assays. The stimulation of PKA activity by *N. n. atra* PLA_2 is probably not related to the production of FFAs since exogenous AA did not have any effect at 20 μM (Fig. 1) which far exceeds the estimated concentrations of FFAs (4.9 to 6.1 μM) produced by 15 or 50 nM *N. n. atra* PLA_2. The stimulation of PKA and CaM-kinase II by *N. n. atra* PLA_2 in synaptic plasma membrane may be the result of PLA_2-dependent disruption of the membrane structure. We previously found that treatment of synaptic plasma membrane with *N. n. atra* PLA_2 decreased membrane fluidity (Ghassemi A and Rosenberg P, unpublished observations) and altered the activity of synaptic plasma membrane-bound Na^+/K^+-ATPase [21].

It has been reported that approximately 90% of the Ca^{2+}-dependent and Ca^{2+}-independent CaM-kinase II activities are associated with the particulate fraction in lysed synaptosomes and that there is a cross-talk between PKC and CaM-kinase II in intact PC12 cells [23]. We, therefore, tested the possibility of cross-talk between these two kinases in synaptic plasma membrane and found that neither a potent PKC activator, PMA, nor an inactive phorbol ester, α-PDD, had any effects on CaM-kinase II activity (Fig. 2), suggesting that there is no direct PKC-dependent phosphorylation of CaM-kinase II at its autophosphorylation site.

Cytosolic PKC was slightly, but significantly, stimulated by treatment with *N. n. atra* PLA_2 (Fig. 3), and this stimulation was further enhanced by the presence of calyculin A, a potent phosphatase-1 and -2A inhibitor (Fig. 4), indicat-

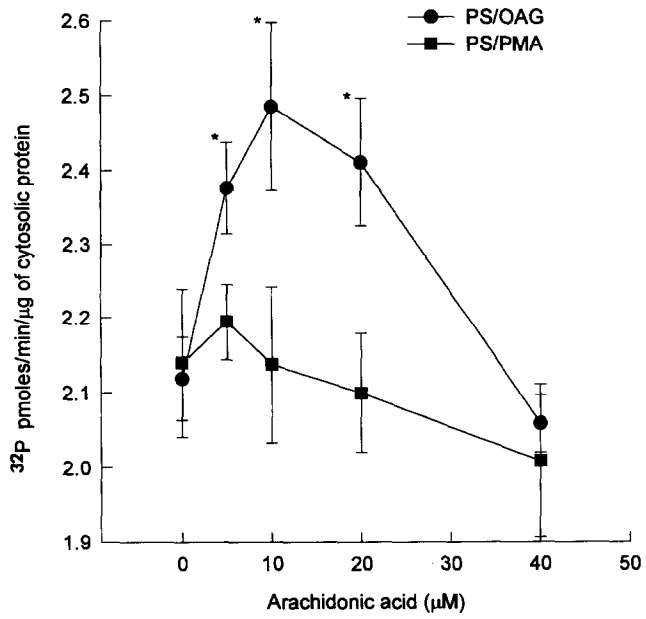

FIG. 5. Concentration-dependent effect of AA on PKC activity in cytosolic fraction. Cytosolic fraction (2 μg protein) was incubated at 30° for 30 min in phosphorylation buffer (50 μL) containing 1 mM $CaCl_2$ and either multilamellar liposomes, composed of 10 μg/mL PS and 1 μg/mL OAG, or 0.5% (v/v) Triton X-100 mixed micelles, composed of 280 μg/mL PS and 10 μM PMA, in the presence of various concentrations of AA. Data are shown as the means ± SEM from three separate experiments. Key: (*) significantly different from control ($P < 0.01$).

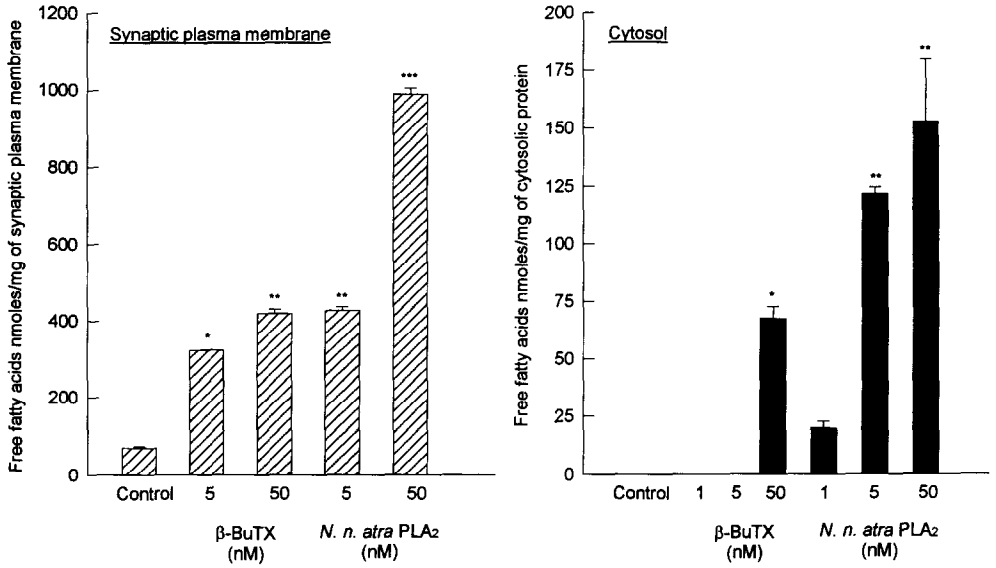

FIG. 6. Production of FFAs by *N. n. atra* PLA_2 and β-BuTX in synaptic plasma membrane and cytosol. Synaptic plasma membrane (1.5 mg protein/mL) or cytosol (0.5 mg protein/mL) in incubation buffer (3 mM $CaCl_2$, 0.9 mM EGTA, Tris-HCl, pH 7.4) were pretreated with *N. n. atra* PLA_2 or β-BuTX at 30° for 30 min. After incubation was stopped by adding 3 mM EGTA, the pretreated synaptic plasma membrane (40 μg protein) or cytosol (13 μg protein) was subjected to FFA measurement. Bars with asterisks were significantly different from control and bars with different numbers of asterisks are significantly different from each other ($P < 0.0001$).

ing that stimulation of PKC-dependent phosphorylation by *N. n. atra* PLA_2 is not due to an inhibition of phosphatase activity. It has been reported that *cis*-unsaturated fatty acids including AA (at effective concentrations of 6–40 μM) stimulates PKC synergistically with diacylglycerol in the presence of micromolar levels of Ca^{2+} [20, 24, 25]. In fact, exogenous AA (5–20 μM) stimulated PKC even in the presence of millimolar levels of Ca^{2+} (Fig. 5). As shown in Fig. 6, treatment of the cytosolic fraction with *N. n. atra* PLA_2 (5 and 50 nM) generated large amounts of FFAs which are mainly considered to be *cis*-unsaturated [5]. Under the conditions of our phosphorylation assay, the estimated final concentrations of FFAs were 4.9 to 6.1 μM. The extent of stimulation of PKC activity by 5 μM AA (113 ± 2%, Fig. 5) was very similar to that caused by *N. n. atra* PLA_2 at 5–50 nM (114–117%, Fig. 3). Our findings that *N. n. atra* PLA_2 did not show a concentration-dependent increase in stimulating PKC activity (Fig. 3) might be explained by the fact that the amounts of FFAs produced in cytosol by 5 and 50 nM were not significantly different (Fig. 6). β-BuTX at 50 nM generated a significant amount of FFAs in the cytosol (Fig. 6); however, it was much less than that of 5 nM *N. n. atra* PLA_2, which may explain why β-BuTX did not have any effects on PKC activity. It is of interest that in synaptic plasma membrane both 50 nM β-BuTX and 5 nM *N. n. atra* PLA_2 had equally low levels of PLA_2 activity (Fig. 6), and at these concentrations both had no effect on PKA or CaM-kinase II activities (Figs. 1 and 2). Likewise, the inability of 50 nM β-BuTX to affect PKC activity (Fig. 3), in contrast to *N. n. atra* PLA_2, correlates with the low levels of PLA_2 activity of β-BuTX in the cytosol (Fig. 6). These results, therefore, suggest that while high levels of PLA_2 activity (FFAs released) may be responsible for some of the effects by *N. n. atra* PLA_2 observed in this study, the low PLA_2 activity possessed by β-BuTX has no direct effects on these kinases.

Our current studies clearly show differences between the effects of *N. n. atra* PLA_2 and those of β-BuTX on the activities of PKA, CaM-kinase II, and PKC. Although we previously found that both β-BuTX and *N. n. atra* PLA_2 inhibit phosphorylation in intact synaptosomes, their mechanisms of action seem to be quite different. In intact synaptosomes, the stimulation of PKA, CaM-kinase II, and PKC activities by *N. n. atra* PLA_2 as observed in this study may be antagonized by its depleting effect on synaptosomal ATP [17], leading to an inhibition of phosphorylation. The inhibitory mechanism of β-BuTX on phosphorylation remains unknown. β-BuTX does not decrease ATP levels [18] nor does it directly inhibit the kinases (the present study). β-BuTX may alter the accessibility of the kinases to their substrates. It is, for example, well known that PKCs are activated upon their translocation from cytosol to membrane and also that every PKC requires PS as the lipid activator [25]. Since β-BuTX dramatically alters the distribution of PS between the inner and outer layers of synaptic plasma membrane [26] we are currently investigating its effect on PKC translocation.

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