PHOSPHOLIPASE A INHIBITION OF ACETYLCHOLINE RECEPTOR FUNCTION IN TORPEDO CALIFORNICA MEMBRANE VESICLES

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SUMMARY. A protein isolated from Naja naja siamensis venom on the basis of its phospholipase A activity inhibits acetylcholine receptor function in postsynaptic membrane vesicles from Torpedo californica. Specifically, the phospholipase A prevents the large increase in sodium efflux that can normally be induced by carbamylcholine, a receptor agonist. The phospholipase A inhibition shows the following properties: 1) it occurs at concentrations 50 times lower than the concentrations required for inhibition by α-neurotoxins; 2) the phospholipase A has no effect on the binding properties of the receptor; 3) the inhibition is abolished by removal of calcium ions; and 4) some phospholipid hydrolysis accompanies inhibition. It is suggested that the phospholipase A acts enzymatically to uncouple ligand binding from ion permeability in the receptor containing membrane vesicles.

The binding of acetylcholine to the acetylcholine receptor results in a change in the ion permeability of postsynaptic membranes (1). Considerable progress has been made in isolating, purifying and characterizing the protein component in electric tissue that specifically binds acetylcholine and other pharmacologically indentified agonists and antagonists of the nicotinic AChR (1,2). However, the mechanism by which ligand binding is coupled to membrane ion permeability is not known, and it is not yet established that the isolated AChR represents a complete functional complex. Efforts to reconstitute AChR function following purification have been at best only partially successful (3,4).

The discovery that functional membrane vesicles enriched in AChR can be prepared from electric tissue of Torpedo species has made it possible to study the biochemical and biophysical properties of the AChR in a membrane environ-

Abbreviations
AChR, acetylcholine receptor; PLA, phospholipase A (EC 3.1.1.4); [^{3}H]MBTA, 4-[N-maleimido]benzyltri[^{3}H]methyl ammonium iodide; Carb, carbamylcholine chloride.

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ment (5,6). The Torpedo membrane vesicles respond to cholinergic agonists by an increase in Na^+, K^+ and Ca^{++} ion permeability. This increased permeability is blocked by specific cholinergic antagonists, such as d-tubocurarine and snake venom α-neurotoxins. Increased ion permeability is also blocked by pretreatment of the membranes with cholinergic agonists, a physiologically important phenomenon known as desensitization (7).

In this paper, the inhibitory effects on Torpedo membrane function of a protein component from Naja naja siamensis venom, isolated on the basis of its PLA activity, are reported. The PLA is being used to investigate lipid-protein interactions in the membranes that might be important for AChR function.

MATERIALS AND METHODS

Torpedo Membrane Vesicles. Live Torpedo californica were obtained from Pacific Biomarine (Venice, CA) and sacrificed immediately upon arrival. Membrane vesicles were routinely prepared from 60 g of fresh electric tissue as described by Hazelbauer and Changeux (4), except that a Brinkmann Polytron was used for homogenization. The procedure was occasionally scaled up to use 500 g of tissue. In this case, the first supernatant was centrifuged at 15,000 x g for 45 min and resuspended in 150 mls of vesicle dilution buffer (255 mM KCl, 1.5 mM $NaPO_4$, 4 mM $CaCl_2$ 2 mM $MgCl_2$, pH 7.0) before the sucrose step gradient.

Assay for AChR Function. The [^{3}H]MBTA affinity labeling assay described by Karlin et al. (8) and/or the binding of tritiated Naja naja siamensis α-neurotoxin (2) were used to determine the number of AChR binding sites in the isolated membranes.

The sodium efflux assay described originally by Kasai and Changeux (9) for Electrophorus electricus membranes was used to determine the functional integrity of the vesicles. Vesicles (5-10 mg/ml) were incubated with ^{22}NaCl (New England Nuclear; 50 μCi/ml) overnight at 4°. At time 0, the vesicles were diluted 100-fold into ice-cold dilution buffer. At time t, 1 ml aliquots were filtered through HAWP 2400 Millipore filters, washed three times with 3.5 mls of ice-cold buffer, dried, and counted in a toluene-based scintillation fluid. Counts retained on the filter correspond to $^{22}Na^+$ trapped within the vesicles.

Fractionation of Naja naja siamensis venom. Lyophilized venom (428 mg, Miami Serpentarium) was dissolved in 5 mls of 0.03 M ammonium acetate (pH 6.5), and purified by ion exchange chromatography on a 1.5 x 30 cm CM-25 Sephadex column. The column was eluted step-wise with increasing concentrations of ammonium acetate (0.03-1.5 M) and protein was monitored at 280 nm with an ISCO UV detector.

All the protein peaks were analyzed for PLA activity. Most of the activity was associated with the peak that did not bind to the CM-25 column in the initial 0.03 M ammonium acetate elution. This crude fraction was further purified by two different procedures: 1. Boiling and gel filtration as described by Cremona and Kearney (10) or 2. anion exchange chromatography on QAE Sepahdex. The QAE Sephadex column was eluted with increasing NaCl concentrations in 0.05 M Tris pH 8.3 essentially as described by Bon and Changeux for Bungarus caeruleus venom (11). The main PLA activity emerged as a skewed peak at 0.1 M NaCl.

Complete details of the purification will be published elsewhere. (M. McNamee, manuscript in preparation).

Assay for PLA Activity. 0.5 μmoles of purified egg phosphatidylcholine was dispersed in 0.3 mls of 10 mM TES--2.5 mM calcium acetate buffer, pH 7.0; 0.1 mls of diethyl ether was then added and at time 0, 10 μl of the sample to be assayed was added. After 5 min at 25°, a 25 μl aliquot was spotted on a silica TLC plate and developed in chloroform:methanolammonia:water (65:25:1:4). The plate was sprayed with a phospholipid spray (Supelco Phospray), and the results expressed as μmoles of phosphatidylcholine hydrolyzed per min per mg protein.

Other Assays and Procedures. Protein was determined by the Lowry procedure (12). Slab SDS-polyacrylamide gels were prepared, run and stained according to the general procedures of Ames (13).

RESULTS

Characterization of Torpedo Membrane Vesicles

From 60 g of fresh tissue, a membrane pellet containing 10-30 mg of protein is obtained. The specific activity of AChR is 800-2500 pmoles of tritiated α-neurotoxin bound per mg protein. If a value of 8,000 pmole/mg is assumed for pure AChR (2), the isolated vesicles contain about 10-30% of their protein as AChR. The number of sites labeled by [^{3}H]MBTA is one-half the number of toxin sites, exactly as observed with purified AChR (2).

The effect of Carb on the efflux of $^{22}Na^{+}$ from the vesicles is shown in Figure 1. The large enhancement in efflux occurs within the first minute and is completely blocked by preincubation with Naja naja siamensis α-neurotoxin (Fig. 1A). Also, the Carb-induced efflux is blocked by prior incubation of the vesicles with 10^{-4} M Carb (Fig. 1B). Thus, these vesicles retain the functional properties characteristic of AChR in intact electroplax (5).

The extent of AChR inhibition by toxin is stoichiometric; i.e., a partial Carb response can be elicited from the vesicles until the number of toxin molecules just exceeds the number of available receptor sites (Figure 2).

Inhibition of $^{22}Na^{+}$ Efflux by PLA

The major PLA containing fraction isolated from N. n. siam. venom by two different procedures (see METHODS) appears as a single band on SDS-polyacrylamide gels corresponding to a MW of ∿18,000 daltons. This protein accounts for about 10% of the total venom protein. In the PLA assay system used here, its specific activity is about 150 Units/mg. Similar specific activities were ob-

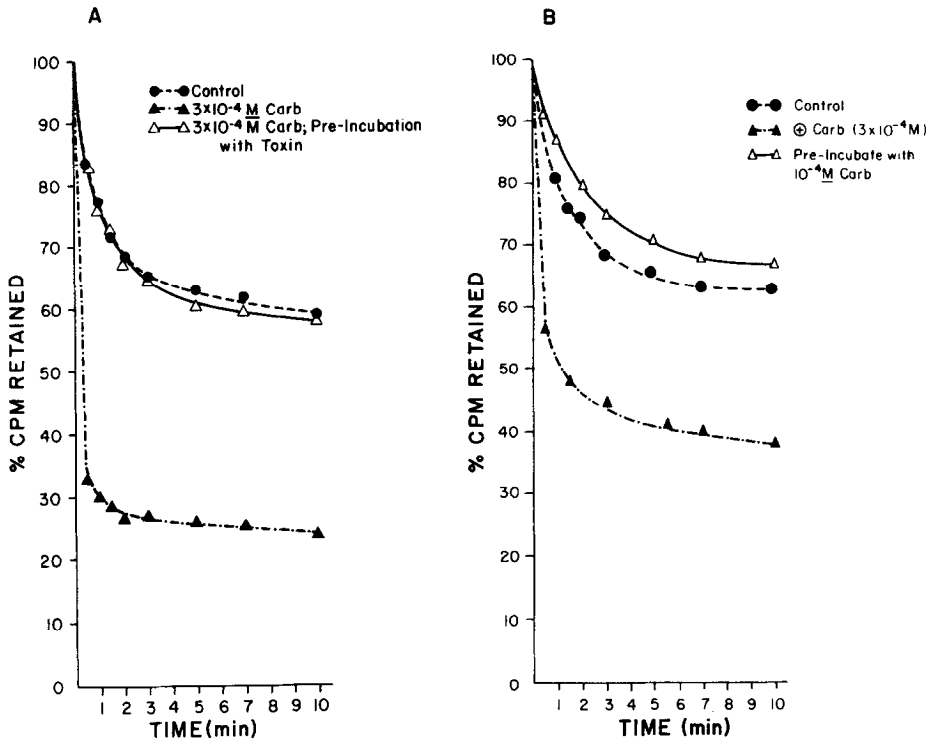

Figure 1. Effect of Carb and Naja naja siamensis α-neurotoxin on $^{22}Na^+$ efflux from Torpedo membrane vesicles. The effect of Carb was measured by including 3 x 10^{-4} M Carb in the dilution medium. (A): Pre-incubation of membrane (5 mg/ml) with α-neurotoxin (180 μg/ml), or (B) pre-incubation with 10^{-4} M Carb for 30 min before dilution into 3 x 10^{-4} M Carb completely blocks the Carb response.

tained for PLA samples obtained commercially (see below).

Pre-incubation of Torpedo membranes (12 mg/ml; ∿10 μM α-neurotoxin sites) with PLA (5 μg/ml) results in complete blockage of the Carb-induced increase in $^{22}Na^+$ efflux within 10 min (Fig. 3A). After longer incubation times, there is a slow increase in passive $^{22}Na^+$ efflux; i.e., the number of counts retained on the filter 1 min after dilution of the membranes into Carb-free buffer gradually decreases (Fig. 3B), presumably due to non-specific vesicle disruption.

Unlike the situation with α-neurotoxin inhibition, the blockage of $^{22}Na^+$ by PLA is not stoichiometric. At a concentration of 5 μg/ml, there is approximately 1 PLA molecule per 33 AChR binding sites.

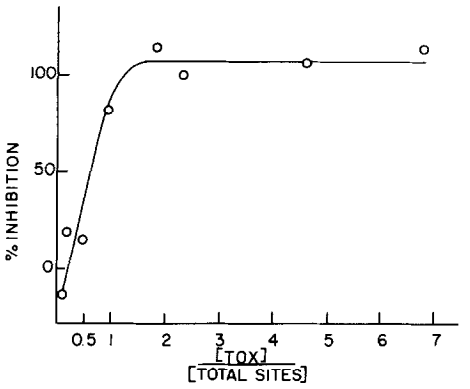

Figure 2. Concentration dependence of α-neurotoxin inhibition. Torpedo membranes were pre-incubated with various concentrations of α-neurotoxin (0-27 μM) for 30 min before dilution into vesicle dilution buffer with and without 2.5×10^{-4} M Carb. The difference after 1 min in $^{22}Na^{+}$ counts between the plus Carb and minus Carb samples is used as a quantitative measure of the Carb response (no difference = 100% inhibition; the difference in the absence of α-neurotoxin = 0% inhibition). The concentration of α-neurotoxin binding sites was 4 μM.

A commercially available Naja naja PLA (Sigma) shows three major bands on SDS gels including one at MW 18,000. This PLA also blocks $^{22}Na^{+}$ efflux. However PLA from Crotalus adamanteus (Sigma) does not block $^{22}Na^{+}$ efflux even at concentrations as high as 100 μg/ml; on SDS-polyacrylamide gels, the Crotalus PLA shows one major band at MW 21,000. There is no detectable proteolytic activity associated with the PLA proteins under the assay conditions used here, as judged by the position and intensity of Torpedo membrane bands on SDS-polyacrylamide gels.

Effect of PLA on AChR Binding Sites

The PLA has no apparent effect on the binding properties of AChR. The extent of α-neurotoxin binding is not altered by either pre-incubation or simultaneous treatment of Torpedo vesicles with PLA, over a concentration range of 0.5-50 μg/ml. The extent and specificity of affinity labeling by [^{3}H]MBTA is also not altered by PLA. The MBTA assay involves a kinetically-controlled reaction sequence (8), so even slight decreases in the affinity of the AChR binding site for MBTA would be detected.

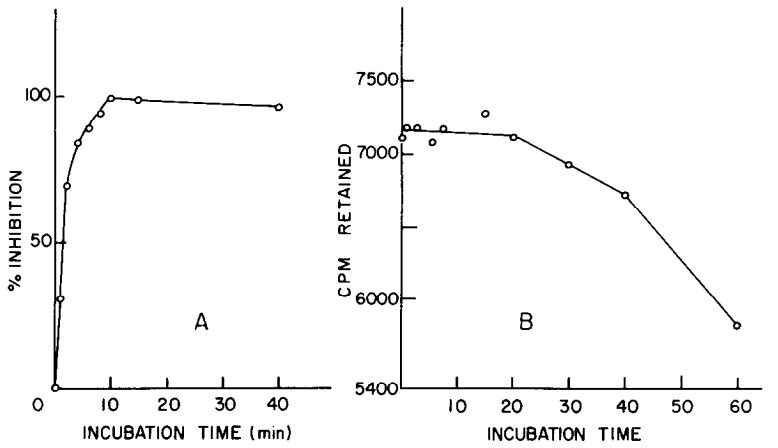

Figure 3. Vesicles were pre-incubated with PLA (5 μg/ml). At the indicated times, 20 μl aliquots of the incubation solution were diluted 100-fold with vesicle buffer (±2.5 x 10^{-4} M Carb) and assayed as described in Figure 2.

A: Inhibition of Carb induced $^{22}Na^{+}$ efflux as a function of incubation time.

B: Increase in passive $^{22}Na^{+}$ efflux as a function of incubation time.

Inhibition of AChR Function by PLA Requires Ca^{++}

PLA is dependent on calcium ions for enzymatic activity. Under normal conditions, the Torpedo membranes are prepared in the presence of 10 mM $CaCl_2$ and are resuspended and diluted into buffer containing 4 mM $CaCl_2$. Vesicles have also been prepared in 0.1 M EGTA and then resuspended in buffers containing Ca^{++}-EGTA so that the free [Ca^{++}] is < 10^{-9} M. Under these conditions, a normal Carb response can be elicited from the vesicles, but this response cannot be inhibited by PLA. The α-neurotoxin, however, still blocks the response. If the free Ca^{++} concentration is increased to 10^{-6} M in the EGTA-containing membranes, PLA inhibition of the carbamylcholine response can be restored.

Lipid Hydrolysis in Torpedo Membrane Vesicles by PLA

In parallel with the $^{22}Na^{+}$ efflux experiments, the effect of PLA on the phospholipids of Torpedo vesicles was monitored under identical conditions. At PLA concentrations of 5 μg/ml (a concentration at which $^{22}Na^{+}$ efflux is completely

blocked), there is detectable lipid hydrolysis (by TLC) of phosphatidylethanolamine during the first 10 min of treatment. At longer times some hydrolysis of phosphatidylcholine can be detected, but all of the phosphatidylethanolamine is hydrolyzed before any phosphatidylcholine hydrolysis is observable. In the absence of free Ca^{++}, there is no detectable lipid hydrolysis.

DISCUSSION

The results demonstrate that a protein from Naja naja siamensis venom, isolated on the basis of its PLA activity, inhibits AChR mediated ion permeability in Torpedo californica membrane vesicles at a concentration 10 to 50 times lower than the concentration required for α-neurotoxin inhibition. The PLA has no effect on the binding properties of AChR, and the inhibitory effect is abolished in the absence of free Ca^{++} ions. These results suggest that the inhibitory effects result from the enzymic activity of the PLA. Phospholipid hydrolysis could result in the disruption of functionally important lipid-protein interactions necessary for proper coupling of ligand binding to AChR mediated ion permeability. Bartels and Rosenberg (14) observed that a crude PLA fraction from cottonmouth moccasin venom inhibited the Carb induced depolarization of isolated eel electroplax at low concentrations (10 μg/ml). This inhibition was not attributed to lipid hydrolysis since none was detected at the low concentrations used. However, in the experiments described here hydrolysis of phosphatidylethanolamine could be detected. It remains to be established if specific lipid hydrolysis is the important event and if it is the resulting lysolipids and fatty acids or the loss of the intact phospholipid that accounts for the inhibition.

It is interesting that not all PLA proteins bring about inhibition of $^{22}Na^{+}$ efflux from Torpedo. This type of result has been reported for the β-bungarotoxin isolated from Bungarus multicinctus. Most of the toxic effects can be attributed to its PLA activity (15), but not all PLA's are neurotoxic. The failure of C. adamanteus PLA to inhibit may indicate a membrane dependent degree of lipid specificity (16). In the original purification of Naja naja siamensis

venom, Karlsson reported that the crude PLA containing fractions were non-toxic in a mouse bio-assay (17). The toxicity of the purified protein used here is now being investigated.

Recently, Bon and Changeux (11) reported that a toxic protein fraction isolated from Bungarus caeruleus venom (ceruleotoxin) inhibited $^{22}Na^{+}$ efflux from Torpedo marmorata vesicles. Their purified protein fraction also showed PLA activity. Experiments are now in progress to determine more precisely the specificity of the PLA protein, its molecular properties, and the relationships between lipid hydrolysis, lipid-protein interactions and AChR function.

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