Phospholipase A_2 Activity and Substrate Specificity of Snake Venom Presynaptic Toxins†

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ABSTRACT: β-Neurotoxins from certain snake venoms are highly specific toxins acting at the presynaptic side of the neuromuscular junction. In this study biochemical aspects of this high specificity have been investigated. When toxins (notexin and *Naja nigricollis* basic phospholipase) act on a mixture of subcellular fractions obtained from brain cortex (synaptosomes, myelin, and mitochondria), the synaptosomal fraction is preferentially attacked and shows the highest release of membrane protein. As seen from isolated fractions, however, even the mitochondria are rapidly and strongly attacked. Examining the phospholipase A_2 activity of the toxin instead of the release of proteins reveals that synaptosomes represent the best substrate. In contrast to nonneurotoxic phospholipases A_2, that from neurotoxin preferentially uses synaptosomal phosphatidylcholine as a substrate when pure phospholipids isolated from subcellular fractions are used. A relationship between the cholesterol/phospholipid ratio and the sensitivity to toxin action in the various subcellular fractions was found. These data suggest that the neurotoxic effect is mainly due to the substrate specificity of the β-neurotoxins. It is suggested that synaptosomal phosphatidylcholine, embedded in a membrane containing a low amount of cholesterol, is a highly specific substrate for β-neurotoxins.

Venoms from certain snakes are known to act on the peripheral nervous system by blocking transmission at the neuromuscular junction. They act also at brain synapses (Wernicke et al., 1974). The venoms, which are known to act on the presynaptic as well as on the postsynaptic side, contain several neurotoxins: γ-neurotoxins, which produce a nondepolarizing postsynaptic neuromuscular block by binding to the nicotinic acetylcholine receptor (Chang & Lee, 1963; Lee & Chang, 1966), and presynaptic toxins (Lee, 1970, 1972).

The presynaptic toxins interfere specifically with the membrane process responsible for the storage and release of transmitter from the motor nerve terminals (Lee & Chang, 1966; Chang et al., 1973; Thesleff, 1977). Notexin and taipoxin, presynaptic toxins isolated and characterized by Halpert & Eaker (1975, 1976), seem to exert their action by abolishing transmitter release (Harris et al., 1973; Kamenskaya & Thesleff, 1974), thus blocking neuromuscular transmission. According to Cull-Candy et al. (1976), a reduction in the number of vesicles precedes the inhibition of transmitter release.

The mechanism by which presynaptic toxins exert their effects is not known. An attractive hypothesis is that they act by means of their always present phospholipase A_2 activity. Thus, β-bungarotoxin has been shown to interfere with the energy metabolism in mammalian brain synapses (Wernicke et al., 1974, 1975; Howard, 1975), resulting in mitochondria uncoupling and a limited maximal rate of respiration. Fohlman et al. (1976) suggested that taipoxin might act by hydrolyzing membranes of synaptic vesicles. According to Strong et al. (1976), the binding of β-bungarotoxin to synaptic membrane could alter the probability that vesicles will fuse with the membrane. The common idea is that phospholipase A_2 activity of presynaptic toxins is responsible for an alteration in membrane processes, leading to an inhibition of transmitter release.

It has been shown that the enzymic activity of pancreatic phospholipase A_2 could be completely abolished by modifying a single histidine residue of this protein (Wolwerk et al., 1974). Some β-neurotoxins [notexin (Halpert et al., 1976) and β-bungarotoxin (Howard & Truog, 1977)] have been altered in the same way. Both neurotoxicity and enzymic activity were essentially abolished upon modification of the amino acid residue, suggesting a connection between those two activities. Why then are presynaptic toxins neurotoxic while other phospholipases A_2 are not? Firstly, one might suppose that the neurotoxic phospholipases A_2 have a very special substrate specificity for some critical phospholipid in the nerve terminal. The other possibility is that presynaptic toxins have a special ability to gain access to the nerve endings. According to the first hypothesis, the neurotoxic phospholipases A_2 should find their best phospholipid substrate at the synapse. Nonneurotoxic phospholipases A_2 should on the other hand bind to any kind of phospholipid encountered and, in so doing, become so diluted as to make them uneffective at the synapse.

Taking this as a working hypothesis, we have investigated whether two presynaptic toxins (notexin from *Notechis scutatus scutatus* and a basic phospholipase from *Naja nigricollis*) act preferentially on nerve endings rather than on other subcellular fractions such as mitochondria, myelin, or crude nuclei pellets isolated from rat brain cerebral cortex.

Materials and Methods

Materials

All the experiments were performed by using rat brain cerebral cortex (Wistar female rats weighing 150–200 g).

Pure presynaptic toxins (notexin and *N. nigricollis* basic phospholipase) were obtained from D. Eaker, University of Uppsala, Sweden.

Phospholipases A_2 (from *Vipera russelli* and bee venoms) and all the chemicals used (unless specified differently in the text) were purchased from Sigma Chemical Co., St. Louis, MO.

Methods

Preparation of subcellular fractions from rat brain cortex was carried out by using a slight modification of the procedure described by Gray & Whittaker (1962). Brain was dissected

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to take away the white substance and homogenized in 0.32 M sucrose, and subcellular fractions were obtained in the following way.

The homogenate was centrifuged (10 min, 1500*g*), and the supernatant (S_1) was saved. The pellet (P_1) was resuspended in the same volume of 0.32 M sucrose and centrifuged again (10 min, 1500*g*), giving P_1' and S_1'. S_1 and S_1' were pooled together and centrifuged at 17000*g* for 30 min. The P_2 pellet obtained was resuspended in 0.32 M sucrose and layered onto a discontinuous sucrose gradient (0.8, 1.2, and 1.6 M). After 2 h of spinning at 82000*g*, four bands were obtained and collected: myelin (0.32 and 0.8 M interface), synaptosomes I (0.8 and 1.2 M interface), synaptosomes II (light synaptosomes) (floating in 0.8 M), and mitochondria (1.2 and 1.6 M interface). The subcellular fractions obtained were characterized by electron microscopy and by determination of marker compounds. Acetylcholinesterase has been determined by using synthetic substrate (acetylthiocholine iodide) and DTNB[1] according to Ellman et al. (1961). Fumarase has been evaluated by using sodium L-malate as substrate according to Racker (1950). Adenosine triphosphatase assays were done by using ATP as substrate by determination of the phosphate liberated by enzymic hydrolysis. The reaction was stopped by addition of trichloracetic acid (final concentration 5%). After centrifugation an aliquot of the supernatant was submitted to phosphate determination by using the reagent described by Somlo (1968). Oligomycin sensitivity was investigated by using the experimental conditions of Somlo (1968) and ouabain sensitivity according to Wheeler et al. (1975).

Acetylcholine was extracted from the subcellular fractions at acidic pH after boiling the samples for 10 min (Hebb & Whittaker, 1958) and was estimated by using a guinea pig ileum bioassay (Blaber & Cuthbert, 1961). Electron microscopy was done after fixation and staining procedures according to Kanaseki & Kodota (1969).

Lipid extraction and purification and determination and purification of phospholipids were performed as described by Guerin & Napias (1978).

Phospholipase A_2 activity was evaluated as follows. A buffered incubation medium consisting of Tris-HCl (pH 8.5) (50 mM), $CaCl_2$ (20 mM), and sodium deoxycholate (2.7 mM) was used. The temperature was either 37 or 4 °C. After incubation of the membrane fraction, phospholipids were extracted. Lysophospholipids formed enzymatically were separated from intact phospholipids and submitted to phosphorus determination. When phospholipase A_2 activity was investigated by using purified phospholipids as substrate, this procedure was slightly modified. Purified phosphatidylcholine was dissolved in diethyl ether. An aliquot of this solution was added to Tris-HCl buffer, pH 8.5 (50 mM), in order to obtain a phospholipid concentration of 1 mg/mL after evaporation of the ether. The phospholipid suspension was then submitted to ultrasonication for 3 min at 4 °C. Sodium deoxycholate and $CaCl_2$ were added (final concentrations: 2.7 and 20 mM, respectively), and the reaction was started by addition of phospholipase A_2 or toxin. The reaction was stopped by addition of 9 volumes of chloroform–methanol (1:2), immediately followed by 3 volumes of NaCl (0.9%). The reaction tubes were vigorously shaken to extract lipids into the chloroform phase, leaving the protein in the water–alcohol phase.

Toxin Incubation Procedure. Toxin, phospholipase A_2, or deoxycholate treatment of subcellular fractions was done in the following way. To 1 volume of fraction (2–4 mg of protein per mL) were added sodium deoxycholate, $CaCl_2$, and Tris-HCl buffer, pH 8.5 (solutions in sucrose of the same molarity as the subcellular fraction considered), so that the final concentrations were 2.7, 20, and 50 mM, respectively. The reaction was started by toxin or phospholipase addition (5 μg/mL final concentration) and was stopped by centrifugation. The pellets obtained were resuspended in buffered sucrose and resedimented.

Control experiments were done in the same way, except that $CaCl_2$, deoxycholate, and toxin were omitted. The effects of $CaCl_2$ and deoxycholate alone, without toxin, were also determined.

Uptake experiments with tritiated GABA were done on intact or toxin-treated synaptosomes according to Nicklas et al. (1973).

Protein determinations were done according to Lowry et al. (1951).

Table I: Protein Distribution between Pellet and Supernatant[a]

sample treatment	*P* (mg ± SD)	*S* (mg ± SD)	*S/P*
control	44.5 ± 1.5	4.7 ± 1.1	0.105
deoxychloate, $CaCl_2$	37.3 ± 1.1	11.1 ± 0.9	0.276
N. nigricollis toxin	26.9 ± 1.7	19.5 ± 2.0	0.725
notexin	28.4 ± 2.5	22.4 ± 2.5	0.788
bee venom phospholipase A_2	29.4 ± 0.5	21.4 ± 0.3	0.737
Vipera venom phospholipase A_2	35.2 ± 1.0	14.8 ± 1.0	0.420

[a] Incubation time 30 min; temperature 30 °C; $n = 5$. *P* = pellet protein recovery; *S* = supernatant protein recovery.

Results and Discussion

Toxin Action on Crude Mitochondrial Pellets

Effect on Total Protein. When the crude mitochondrial pellet (P_2 pellet) was treated with toxin, the prominent effect (Table I) was a high release of protein from the membranous material. Obviously, the detergent treatment and possible endogenous phospholipase activity of the fraction alone cannot explain the phenomenon. Both toxins give an increased release of protein from the pellets.

When the pellet remaining after the toxin treatment was further separated on a sucrose step gradient, bands were obtained corresponding to those representing myelin, synaptosomes, and mitochondria in control experiments. However, the relative amount of protein recovered in the bands was significantly modified after toxin treatment and consisted essentially in a decrease of synaptosomal proteins, which was not the case with other phospholipases A_2 (not shown).

Characterization of Subcellular Fractions. The above experiments and morphological studies indicated that mitochondria and synaptosomes were the main sites of the toxin action. Therefore, we investigated some of the more characteristic enzymes of both fractions. After toxin treatment (Table II), acetylcholinesterase, a synaptosomal protein, fumarase, a mitochondrial protein (located in the matrix), and adenosine triphosphatase, oligomycin-sensitive ATPase (located on the inner membrane of mitochondria), and Na/K-(ouabain-sensitive) ATPase (located on outer membranes), were greatly reduced in the pellet and were present in the supernatant. Though deoxycholate (and $CaCl_2$) accounts for part of this action on both mitochondrial and synaptosomal membranes, the toxins and phospholipases always produce an additive effect. After treatment the levels of acetylcholinesterase and fumarase in the pellets were dramatically decreased, and that of the oligomycin-sensitive ATPase was only

[1] Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GABA, γ-amino-*n*-butyric acid; AcChE, acetylcholinesterase; ATPase, adenosine triphosphatase; FUM, fumarase.

Table II: Relative Distribution of Marker Enzymes between Pellet and Supernatant[a]

marker enzymes (units)	control	deoxycholate, $CaCl_2$	toxins: *N. nigricollis*	toxins: notexin	phospholipases A_2: bee	phospholipases A_2: *Vipera*
AcChE						
S	0.41	0.63	3.50	3.80	3.10	1.70
P	3.72	4.05	0.50	0.45	1.00	2.10
S/P	0.11	0.15	7.00	8.40	3.10	0.8
FUM						
S	0.015	0.048	0.115	0.120	0.134	0.128
P	0.132	0.070	0.012	0.008	0.013	0.016
S/P	0.11	0.68	9.58	15.00	10.00	0.100
ATPase						
S	0.047	0.052	0.107	0.137	0.33	0.08
P	1.73	1.83	0.33	0.38	0.60	0.74
S/P	0.027	0.028	0.32	0.36	0.54	0.10
oligomycin sensitivity in the pellets	60%	40%	0%	0%	52%	54%

[a] AcChE = acetylcholinesterase; FUM = fumarase; ATPase = adenosine triphosphatase. Units: 1 unit of acetylcholinesterase hydrolyzes 1 μmol of acetylcholine per min; 1 unit of adenosine triphosphatase hydrolyzes 1 μmol of ATP per min; 1 unit of fumarase corresponds to a change of 1.0 in optical density using the experimental conditions described under Methods. $n = 4$.

30–50% and oligomycin sensitivity disappeared after neurotoxin treatment only.

The results demonstrate that the mitochondrial membrane is damaged, as the protein conferring oligomycin sensitivity is lost from the pellet. The recovery of acetylcholinesterase and fumarase in the pellet and supernatant is total, while the recovery of ATPase is only 25–30%. The toxin action is correlated with decreases in specific activities on the tested enzymes from the pellet (not shown). In the case of acetylcholinesterase and fumarase, one can correlate the decreases in specific activities of the pellets with a high release into the supernatant. Toxins and nontoxic phospholipases have a strong action on mitochondrial membranes, as shown by fumarase release in the supernatants (Table II). For adenosine triphosphatase this cannot be the case. Oligomycin sensitivity is not affected by detergent treatment but is greatly reduced, especially in the mitochondrial fraction, after toxin action. The activity of this protein is known to be lipid dependent, and such a decrease would reflect changes in the lipid environment of the enzyme. This can also explain the apparent loss of total activity. Of importance is the action of nonneurotoxic phospholipases A_2. Bee venom phospholipase always showed strong effects but a lower release of AcChE than neurotoxins, which indicates the lesser extent of attack of synaptic membranes. *Vipera* venom phospholipase has a very weak effect on synaptic membranes but strongly attacks mitochondrial membranes as shown by the *S/P* ratio of fumarase. It is also of importance to point out that neither of the nonneurotoxic phospholipases A_2 had an effect on the oligomycin sensitivity of ATPase. The total activity of this enzyme was also reduced due to lipid changes in the pellets. The release of enzymes, only partially due to the deoxycholate present in the medium, confirmed that the presynaptic toxin does considerable damage to synaptosomal and mitochondrial membranes.

Toxin Action on Isolated Subcellular Fractions

The results obtained with crude mitochondrial pellets thus indicated that the effect of the toxin appeared to be directed toward a specific target, probably the synaptosomes, but mitochondria seemed to suffer strong attack as well. This was studied further on sucrose gradient subfractions.

Effect on Total Protein. As seen in Figure 1, protein from mitochondria and synaptosomes was released into the supernatant much more quickly than that from myelin.

In a separate experiment carried out at 4 °C instead of 37 °C and using a protein concentration of 1 mg/mL, the apparent velocity of release of proteins from the pellet into the supernatant was respectively 0.26 mg of protein per min for mitochondria, 0.41 mg of protein per min for synaptosomes, and 0.08 mg of protein per min for myelin. When the toxin treatment was done at a higher temperature and for a longer time, the percent of protein released reached the same value in the various subcellular fractions (not shown). Thus, the toxins used attack all subcellular fractions tested, but at different rates. As the toxins exhibit phospholipase A_2 activity toward these fractions, the difference in initial velocity of attack might have correlated with the content of phospholipids in the various fractions. This was not the case. The myelin fraction, which was attacked with the lowest initial velocity, had the highest phospholipid/protein ratio (Table III). Further, quantitative separation of phospholipids by TLC (Figure 2) indicated that the relative amount of phosphatidylcholine + phosphatidylethanolamine did not vary significantly among the different fractions. Therefore, the specific attack of the toxin could not be attributed to differences in the quantity of substrate.

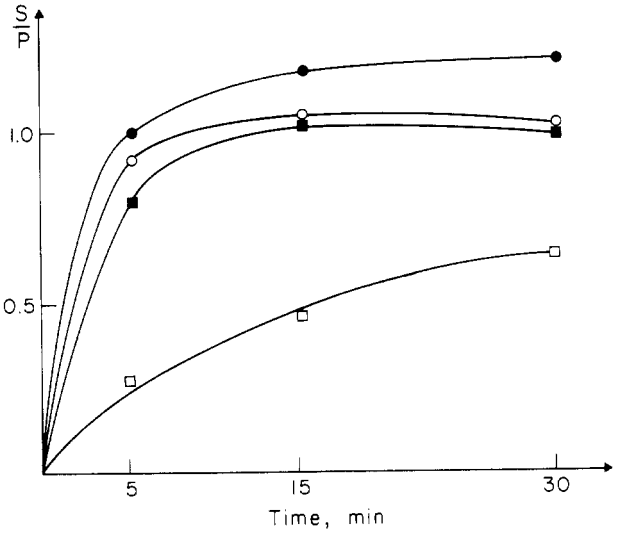

FIGURE 1: Time course of β-neurotoxin (notexin) action on the various isolated subcellular fractions: (□) myelin; (■) mitochondria; (○) synaptosomes I (heavy synaptosomes); (●) synaptosomes II (light synaptosomes).

Effect on Marker Enzyme Content. Table IV shows that the specific proteins studied were released from their sources

Table III: Phospholipids and Cholesterol Contents in the Various Subcellular Fractions

	mitochondria	synaptosomes	crude nuclei pellet	myelin
phospholipids [[mg/(mg of protein)] × 100]	26.10 ± 0.60	29.40 ± 2.80	35.10 ± 0.70	63.10 ± 5.20
cholesterol [[mg/(mg of protein)] × 100]	2.77 ± 0.44	5.71 ± 0.51	11.76 ± 2.31	33.28 ± 5.05
cholesterol/phospholipids (mol/mol)	0.21 ± 0.03	0.39 ± 0.05	0.67 ± 0.13	1.05 ± 0.19

Table IV: Release of Total Proteins and Marker Enzymes from the Pellets of Isolated Subcellular Fractions[a]

	mitochondria		synaptosomes I		synaptosomes II		myelin	
marker enzymes (units)	control	toxin	control	toxin	control	toxin	control	toxin
AcChE	0.36	0.016	2.60	0.17	2.10	0.074	0.86	0.090
FUM	0.56	0.001	0.64	0.09	0.40	0.075	0.08	0.020
ATPase	0.70	0.100	0.31	0.18	0.21	0.070	0.13	0.045
oligomycin sensitivity	80%	0%	68%	2%	60%	40%	20%	
ouabain sensitivity	30%	100%	45%	60%	50%	60%	90%	
total proteins	100%	48%	100%	45%	100%	41%	100%	55%

[a] Toxin was *N. nigricollis* toxin, incubation time 30 min, and temperature 37 °C. Enzyme units could vary from one experiment to another, depending on the total proteins and on the physiological state of the material. Consequently, this table gives the values obtained from one model experiment.

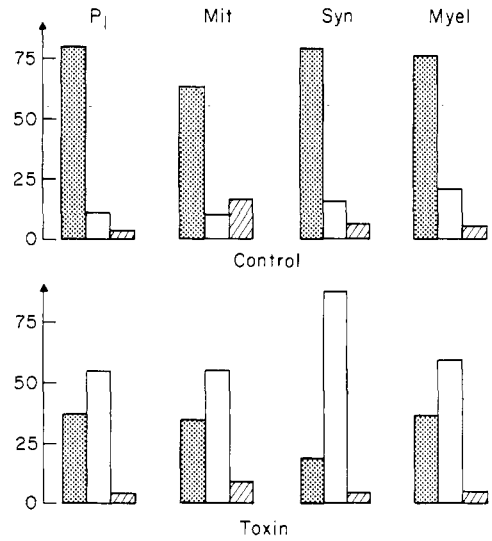

FIGURE 2: Relative amounts of phospholipids among the various isolated subcellular fractions before and after toxin action (notexin). Temperature = 30 °C, and toxin action (Methods) was for 15 min. S/P = (protein in the supernatant)/(protein in the pellet). (Dotted) Phosphatidylethanolamine + phosphatidylcholine; (open) sphingomyelin + phosphatidylserine + lysophosphatidylcholine; (hatched) phosphatidylinositol + phosphatidic acid + other polar phospholipids.

and that the release did not parallel the release of total protein. This result confirmed those obtained with crude synaptosomal pellets. In the case of the adenosine triphosphatase activity, we emphasize that the loss in oligomycin sensitivity was associated with an increase in ouabain sensitivity, again indicating that the membranes of mitochondria and synaptosomal mitochondria were severely damaged by the toxin.

Synaptosomal GABA Uptake. Studies of GABA uptake showed, as was expected from the morphological studies, that toxin-treated synaptosomes were not able to incorporate the transmitter. The amount of GABA uptake was never more than the "noise value" (not shown).

Toxin Specificity. The results given above indicated that both mitochondria and synaptosomes are good targets for the toxins studied. This observation does not take into account the fact that mitochondria might be more "fragile" than synaptosomes; in such a case, only a slight attack could lead to a high degree of destruction. We compared the ratio of protein in the supernatant to protein in the pellet (called the destruction factor) with the ratio of lysophospholipid formed to phosphatidylcholine left (called the substrate quality factor)

Table V: Toxin Specificity toward Various Isolated Subcellular Fractions[a]

	mitochondria	synaptosomes	crude nuclei pellet	myelin
destruction factor	1.46	1.50	0.68	0.44
substrate quality factor	1.10	9.80	1.40	1.60

[a] The toxin used in these experiments was *N. nigricollis* toxin. Incubation time was 30 min, and temperature was 4 °C. The numbers represent the values obtained in one model experiment. No mean value is given, since both factors studied showed large variations from one experiment to the other (10% for destruction factor and 20-30% for the substrate quality factor). $n = 5$ for destruction factor; $n = 3$ for substrate quality factor.

obtained after toxin treatment (Table V). The reaction rate was linear for all the fractions under the experimental conditions described elsewhere. One can see that both mitochondria and synaptosomes showed similar high destruction factors as compared with other fractions such as myelin or crude nuclei pellets. If we look at the substrate quality factor, synaptosomes represented the best substrate for toxin phospholipase A_2 activity, whereas mitochondria were rather a poor substrate. This result supports the other evidence that mitochondria are in fact more sensitive to destruction (high destruction factor) than other subcellular fractions, not because they are especially good targets for the toxin action but because they are more "fragile" than synaptosomes. These findings should be correlated to a binding study of β-bungarotoxin on different membrane preparations (Oberg & Kelly, 1976). The authors showed that β-bungarotoxin binds much more to brain membranes than to other kinds of membranes, such as liver cell or red blood cell membranes. The authors pointed out this specific binding of an enzyme showing high affinity and discriminatory power toward different kinds of membranes.

Phosphatidylcholines from the various subcellular fractions were purified by thin-layer chromatography in order to clarify this important point. We compared the substrate quality factors of the purified phosphatidylcholines toward nonneurotoxic and neurotoxic phospholipases A_2 (Table VI). Obviously, synaptosomal phosphatidylcholine is a good substrate for the neurotoxin. It is of major interest to note that bee venom phospholipase A_2 shows a behavior half way between the two other phospholipases A_2. It is known that bee venom is centrally neurotoxic. However, this toxicity is mainly due to apamin (Habermann & Fischer, 1979a,b). Our results tend

Table VI: Substrate Specificity of Different Phospholipases A_2[a]

enzyme	source of phosphatidylcholine used: mitochondria	synaptosomes	crude nuclei pellet	myelin	N.B.C.
N. nigricollis toxin	1.0	4.5	2.3	5.7	6.3
phospholipase A_2 (bee venom)	0.6	1.2	1.3	10.0	8.0
phospholipase A_2 (*Vipera* venom)	3.2	2.2	1.4	10.5	8.2

[a] N.B.C. = phosphatidylcholine purchased from N.B.C. (synthetic palmitylpalmitoleylphosphatidylcholine). Numbers represent the substrate quality factors of the various phosphatidylcholines compared to the substrate quality factor of mitochondrial phosphatidylcholine taken as unit, when neurotoxin is used. Temperature was 30 °C, and incubation time was 15 min. Numbers were obtained from two separate experiments in which the phospholipid determinations were done in triplicate. Hydrolysis of the substrate was less than 30%, and the reaction rate was linear for all tested phosphatidylcholines except myelin. Substrate purity was more than 95% when checked by phosphorus determination after two-dimensional thin-layer chromatography. Recoveries of phosphatidylcholine and lysophosphatidylcholine were 100 ± 5%.

to support the evidence that phospholipase A_2 could also be neurotoxic, at least, on the peripheral nervous system. Thus, in the venom, phospholipase A_2 could act synergically with apamin and potentiate its action. Mitochondrial phosphatidylcholine is a good substrate for *Vipera* venom phospholipase A_2 but not for the other phospholipases A_2. On the other hand, it appears that myelin phosphatidylcholine and a commercial synthetic product (palmitylpalmitoleyl-L-α-phosphatidylcholine) are always the best substrates of those tested for any kind of phospholipase A_2.

These results tend to support the evidence that the phospholipase A_2 activity of presynaptic toxin has a characteristic substrate specificity since synaptosomal phosphatidylcholine is one of its best substrates. Sen et al. (1976) reported that a phospholipase A_2 inhibitor did not inhibit the neurotoxic effect of β-bungarotoxin, which inhibits synaptosomal choline uptake and reduces the synaptosomal store of acetylcholine. However, Ronald & Howard (1977) have more recently shown that Sen et al. (1976) did not actually measure the phospholipase A_2 activity of β-bungarotoxin with the assay used and furthermore that the competitive inhibitor used was not inhibitory at the concentration employed. Therefore, one must be very careful in the choice of experimental conditions used for phospholipase A_2 activity measurements. Apparent discrepancies are often due to differences between the methods used.

We have not investigated the effect of phospholipase inhibitors. From our studies the specificity of presynaptic toxin does not seem to be directed toward the polar head of the phospholipid. This specificity toward molecules having the same hydrophilic part must be largely dependent on the hydrophobic part. A study of the fatty acids of the various phosphatidylcholines might provide us with important information on this point. The cholesterol content of the various subcellular fractions might also play a role in this specificity. Recently, Strong & Kelly (1977) have shown that the phospholipase A_2 activity of β-bungarotoxin is largely dependent on the physical state of the phospholipid. They clearly demonstrated that β-bungarotoxin prefers to hydrolyze membranes undergoing phase transition or containing a solid–fluid phase boundary. The addition of cholesterol (compound eliminating the phase transition of phospholipids) to synthetic phospholipid inhibits the phospholipase activity of β-bungarotoxin. As shown in Table III, our results are in good agreement with these findings. Synaptosomes and mitochondria, the fractions having the lowest cholesterol/phospholipid ratio, are the best substrates.

Our results do not exclude the existence of a specific protein located at the synapse and showing a very high affinity for the presynaptic toxin. This protein should be surrounded by phospholipids and especially phosphatidylcholine and would provide a sort of phase boundary. If the postulated protein–lipid association was tight enough, one might be able to isolate the complex in a lipid-soluble state. Further work could be undertaken to test such a hypothesis.

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Human Plasma Prekallikrein. Studies of Its Activation by Activated Factor XII and of Its Inactivation by Diisopropyl Phosphofluoridate†

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ABSTRACT: Human plasma prekallikrein was purified from normal plasma. The purified prekallikrein appeared homogeneous on polyacrylamide gels in the presence of sodium dodecyl sulfate and mercaptoethanol and gave two protein bands with approximate M_r 85 000. Proteolytic activation of prekallikrein by purified human β-factor XII_a (M_r 28 000 form) resulted in the formation of kallikrein. The apparent molecular weight of kallikrein determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the absence of mercaptoethanol was identical with that of prekallikrein; reduction of kallikrein yielded a heavy chain of M_r 52 000 and two light chains of M_r 42 000 and 37 000. The appearance of kallikrein activity was directly correlated with the limited proteolysis due to β-factor XII_a. Kinetic and immunologic studies demonstrated that plasma prekallikrein is a factor XII dependent plasminogen proactivator. The rate constant for the inactivation of prekallikrein by diisopropyl phosphofluoridate was similar to that previously reported for trypsinogen. This observation raises the possibility that low intrinsic catalytic activity of prekallikrein may play a role in the initiation of the intrinsic blood coagulation pathway.

Kallikrein is a serine protease that effects the release of vasodepressor peptides or kinins (including bradykinin) from kininogens. These kinins can also increase vascular permeability, contract smooth muscles, produce pain, and influence the migration of leukocytes (Erdos, 1966). Kallikrein can be derived from tissue or from plasma (Kraut et al., 1928), and the plasma enzyme circulates as an inactive zymogen, prekallikrein.

Fletcher trait plasma (Hathaway et al., 1965; Hathaway & Alsever, 1970) which possessed abnormalities in the intrinsic coagulation pathway and in the generation of kinins and of kaolin-activated fibrinolytic activity (Saito et al., 1974; Weiss et al., 1974) was shown to be deficient in prekallikrein (Wuepper, 1973).

Plasma prekallikrein participates in the initiation of the intrinsic pathway of blood coagulation. After exposure of plasma to a negatively charged activating surface such as kaolin or glass, surface-bound factor XII (Hageman factor) undergoes a conformational change resulting in a structure that is highly susceptible to proteolytic cleavage, and this proteolytic cleavage is mainly responsible for the activation of factor XII (Griffin & Cochrane, 1976a; Revak et al., 1977; Griffin, 1978). Surface-bound factor XII_a in the presence of high molecular weight kininogen is a potent activator of factor XI and of prekallikrein (Griffin & Cochrane, 1976a; Revak et al., 1978). The newly formed kallikrein readily dissociates from the surface (Wiggins et al., 1977; Cochrane & Revak, 1979) and reciprocally activates more surface-bound factor XII, thereby augmenting the amount of factor XII_a and consequently the activation of factor XII and prekallikrein. Prekallikrein (Mandle et al., 1976; Donaldson et al., 1977) and factor XI (Thompson et al., 1977) exist in normal plasma complexed with high molecular weight kininogen. This high molecular weight kininogen links both factor XI and prekallikrein to a negatively charged surface where they are activated by surface-bound α-factor XII_a[1] (Wiggins et al., 1977). Once activated, the factor XI_a molecules remain localized at the site of activation, in contrast to the kallikrein molecules which dissociate into the surrounding space (Wiggins et al., 1977; Cochrane & Revak, 1979).

The initial events in triggering the reciprocal proteolytic activations of factor XII and prekallikrein are still unclear. However, on the basis of the observations that trypsinogen and chymotrypsinogen possess weak intrinsic activity in reactions with diisopropyl phosphofluoridate as well as with ester and

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[1] Abbreviations used: factor XI_a, the active procoagulant form of factor XI; α-factor XII_a, the active, two-chain M_r 80 000 form of factor XII; $NaDodSO_4$, sodium dodecyl sulfate; β-factor XII_a, the active M_r 28 000 form of factor XII; DFP, diisopropyl phosphofluoridate.

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