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DEMONSTRATION OF PHOSPHOLIPID SPLITTING AS THE FACTOR RESPONSIBLE FOR INCREASED PERMEABILITY AND BLOCK OF AXONAL CONDUCTION INDUCED BY SNAKE VENOM

I. STUDY ON LOBSTER AXONS

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SUMMARY

1. Axons from the walking leg of lobster have been treated with snake venoms and with isolated phospholipase A and direct lytic factor. Changes in axonal conduction, in sensitivity to curare and acetylcholine and in phospholipid composition have been determined.

2. The block of electrical activity induced by high concentrations of ringhals and moccasin venoms and the facilitated penetration of curare and acetylcholine in axons treated with lower amounts of the above venoms were each associated with a certain level of phospholipid splitting. The isolated venom phospholipase A fraction reproduced the effects of whole venom.

3. In contrast to ringhals and moccasin venoms, palestinian viper and rattlesnake venoms were unable to block axonal conduction and did not markedly affect the axonal phospholipids. In the conditions of the present study their action was not potentiated by addition of the venom direct lytic factor.

4. The data are considered as evidence for phospholipase A being the factor in venoms responsible for inducing block of conduction and increased permeability in lobster axons.

INTRODUCTION

Over the past several years the effects of various venoms on the permeability and function of the squid giant axon have been studied[1–8]. It has been shown that cottonmouth moccasin and other venoms in relatively low concentrations, which do not markedly affect conduction, will increase the penetration of many compounds which otherwise do not enter the cell interior. Higher concentrations of these venoms will irreversibly block conduction, apparently acting on the excitable membrane.

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Compounds such as acetylcholine and curare which have potent effects at synapses do not normally affect axonal conduction even though as postulated by NACHMANSOHN[9,10] acetylcholine is essential for conduction. It was suggested that this failure of action was due to the existence of structural barriers preventing the compounds from penetrating to the axolemma[3,11]. Following pretreatment of the axon, with concentrations of venom which did not affect the action potential, acetylcholine and curare penetrated and reversibly blocked conduction. The permeability to acetylcholine was also markedly increased in axons from the walking leg nerves of lobster and the concentrations of acetylcholine required for affecting conduction were markedly decreased by venom pretreatment[12,13].

In these studies marked variation in potencies of venoms was observed. Certain venoms such as cottonmouth moccasin and hooded cobra were highly potent when used on the squid axon, whereas others, such as russels viper and eastern diamondback rattlesnake, were relatively inactive both in their direct effects on conduction, and in their ability to render the axons sensitive to curare and acetylcholine[2,4,8]. On the basis of various experiments it appeared that phospholipase A might be the active venom component of cottonmouth venom[2,5]. Venom effects on permeability and function of other membranal systems have also been reported and have been attributed to their phospholipase A content[14,15]. However, the differences in phospholipase A activities of cottonmouth and rattlesnake venoms compared on a semipurified preparation of egg or beef heart lecithin as substrate did not account for the marked differences in potency of these venoms[5]. A possible explanation is suggested by the accumulation of data on the difference in ability of phospholipases A from various snake venoms to hydrolyze phospholipids in biological membranes. Such differences between Cobra and *Vipera palestinae* phospholipase A have been demonstrated on red cell ghosts[16,17], platelets[18], and mitochondria[19]. Moreover, an additional venom fraction, the direct lytic factor was shown to facilitate the attack of venom phospholipases A on membrane-bound phospholipids. The presence of this factor in Cobra venoms in contrast to its absence in *V. palestinae* venom accounts for the difference in the effects of these venoms on human and animal erythrocytes[20].

The aim of the present study was to determine if phospholipase A is responsible for the venom effects observed on axons, and if so, to find a possible explanation for the differences found between the venoms. Phospholipase A and direct lytic factor have been isolated from venoms and these isolated fractions as well as the venoms have been tested as to their effects on electrical activity of axons from the walking legs of lobsters and on the permeability of this preparation as judged by sensitization to acetylcholine and curare. We also have analyzed changes in axonal phospholipids produced as a result of these various treatments and have compared changes in phospholipid composition with changes on conduction and permeability. In a subsequent paper we shall report on similar studies carried out with the squid giant axon.

MATERIALS AND METHODS

Venoms and chemicals

Viper *(V. palestinae)* venom was obtained through the courtesy of Dr. E. Kochwa (Dept. of Zoology of the Tel Aviv University). Ringhals *(Haemachatus*

haemachatus) venom was purchased from Pierce Chemical Co., Rockford, Ill. Eastern diamondback rattlesnake *(Crotalus adamanteus)* and cottonmouth moccasin *(Agkistrodon p. piscivorus)* venoms were purchased from Ross Allen Reptile Institute, Silver Springs, Fla. Phosphatidyl serine, phosphatidyl inositol and a mixture of lecithin, spingomyelin and lysolecithin were a gift of Dr. M. M. Rapport from the Albert Einstein College of Medicine, N.Y. Phosphatidic acid (sodium salt) was purchased from General Biochemicals, Chagrin Falls, Ohio, and phosphatidyl ethanolamine from Applied Science Laboratory, State College, Pa. Fiske–SubbaRow reagent was purchased from Fisher Scientific Co.

Venom fractionation and measurement of phospholipase A activity

Electrophoresis of the ringhals venom, location, elution and identification of the phospholipase A fraction and of the direct lytic factor have been described[16]. No direct lytic factor fraction was present in rattlesnake venom. The isolated phospholipase A fraction was heated in a boiling-water bath for 15 min at pH 5.5, the pH was then readjusted to 7.0 and both the phospholipase and the direct lytic factor fractions were extensively dialyzed against artificial sea water having the following composition in mmoles/l: NaCl, 423; KCl, 9.00; $CaCl_2 \cdot 2H_2O$, 9.27; $MgCl_2 \cdot 6H_2O$, 22.94; $MgSO_4 \cdot 7H_2O$, 25.50; $NaHCO_3$, 2.15; and Tris, 1.00 (pH adjusted to 7.5–8.0). The slight precipitate appearing during dialysis was removed by centrifugation and protein content of the supernatant determined according to the method of LOWRY *et al.*[21]. Phospholipase A activity was determined by titrating according to the method of DOLE[23] the amount of free fatty acids liberated from egg yolk, diluted 1:10 v/v in physiological saline solution. Whole venoms and phospholipase fractions were thus compared as to their catalytic rates on egg yolk phospholipids.

Venom treatment of lobster axons

Nerves from the walking legs of lobster *(Homarus americanus)* were rapidly blotted on filter paper and the four nerves from the legs on one side of the trunk were pooled for venom treatment and phospholipid analysis. The nerves from the opposite side served either as controls or were used for measurements of electrical activity. Incubation with venoms and venom fractions was carried out on intact and homogenized tissues. Intact nerves weighing 200–400 mg were placed in centrifuge tubes containing venoms or venom fractions dissolved in 2 ml of the artificial sea water described. All incubations of venom with nerve were carried out under pH conditions assuring optimal survival of the axons (pH 7.5–8.0). No study of the effect of pH on venom action was undertaken because of the direct effects of pH changes on electrical activity and because the optimal pH for phospholipase A action of various venoms does not differ greatly[22]. Incubation was routinely carried out at room temperature for 30 min with occasional shaking. At the end of incubation the tubes were centrifuged, the supernatant fluid removed with a pipet, and the tissue washed twice by resuspension in 0.5 ml sea water. The supernatant and washing fluids were pooled. Control nerves incubated without venom were treated similarly. With some nerves a homogenized suspension was prepared with a Tenbroek tissue grinder and incubated in sea water solutions with or without venom in the same way as described for intact tissues.

Extraction of lipids

Lipids were separately extracted from the intact nerves, the pooled supernatant fluid and washings of the intact nerve, and from the nerve homogenates. Tissue lipids were extracted by homogenization with chloroform–methanol (2:1, v/v), and the extract washed with 0.04% $CaCl_2$, as described by FOLCH, LEES AND SLOANE-STANLEY[24]. The washed extract was evaporated to dryness under a nitrogen stream and redissolved in 1 ml of chloroform–methanol (2:1, v/v). The pooled supernatants and the homogenates were extracted with methanol–chloroform (3:1, v/v and 1:1, v/v) as described by MARINETTI *et al.*[25] for plasma phosphatides with the following modification: after the extract was evaporated to dryness under nitrogen, the residue was reextracted with chloroform–methanol (2:1, v/v) and washed according to FOLCH, LEES AND SLOANE-STANLEY[24]. The final volume of the homogenate extract was adjusted to 1 ml, and the supernatant extract to 0.5 ml.

Thin-layer chromatography of phospholipids

Plates were prepared by slurring 40 g of silica gel HR (Merck, Darmstadt) with 100 ml of 1 mM Na_2CO_3 solution[26], and applying the mixture in a 0.5-mm layer by means of an adjustable Desaga applicator. The plates were dried in air and activated at 110° for 1 h, before use. Lipid extracts containing approximately 20 μg P were applied, under a stream of N_2, in a spot about 3 cm from the bottom and left edge of the plate. All extracts were run in duplicate. Attempts to separate in a one-dimensional system the individual phospholipids from each other and from their lyso products (arising by venom action) were unsuccessful. The following procedure using two-dimensional chromatography allowed separation of the components with the least degree of overlapping. The plates were first run in solvent system I: chloroform–methanol–water (65:25:4, v/v), until the front approached the edge of the plate. The plates were then dried in a vacuum dessiccator or in an oven preheated to 50°. The solvent system II, 3-heptanone–acetic acid–water (80:50:10, v/v) was used in the second dimension, and run until the front reached the edge of the plate.

After the plates were thoroughly air dried, the spots were revealed with iodine vapor and encircled. For identification we compared the spots with known markers and carried out the following specific color reactions: (a) ninhydrin spray for phosphatidyl ethanolamine, phosphatidyl serine, and the respective lyso derivatives; (b) modified Schiff reagent[27] for plasmalogens, and (c) modified Dragendorff reagent[28] for choline-containing phospholipids. The only overlapping of spots that occurred in the venom-treated samples were phosphatidyl inositol and lysophosphatidyl ethanolamine. However, the amount of lysophosphatidyl ethanolamine produced by the venom action could be calculated by subtracting from the phosphatidyl inositol *plus* lysophosphatidyl ethanolamine spot the amount of phosphatidyl inositol found in the control. That calculation was justified by the observation that the sum phosphatidyl ethanolamine *plus* phosphatidyl inositol in controls was practically equal to the sum phosphatidyl ethanolamine *plus* phosphatidyl inositol *plus* lysophosphatidyl ethanolamine in the venom-treated samples, thus indicating that in our experiments phosphatidyl inositol was not hydrolyzed by the venom phospholipase A. Other studies have also shown that phosphatidyl inositol in tissues is not significantly hydrolyzed by venom phospholipase A[29], although purified

phosphatidyl inositol is slowly hydrolyzed[30]. Depending on amount applied to the plate the commercial sample of phosphatidic acid gave either a large elongated spot or 2 separate spots at places identical to that of the 2 spots consistently found after application of the nerve extract (Fig. 1). We have therefore added the phosphorus content in these 2 spots, recording the total as the phosphatidic acid value, although the actual identity is not certain. We were unable to detect the hydrolytic products of phosphatidic acid, following phospholipase treatment. A similar difficulty was reported by SKIDMORE AND ENTERMAN[31]. The Schiff reagent revealed the presence of plasmalogens in the phosphatidyl ethanolamine, phosphatidyl serine and lecithin spots.

Since the plasmalogen and alkyl ether forms of each of these phospholipids did not separate from their corresponding diacyl derivatives, the term "phosphatidyl' as used in this study refers to the sum of these various forms. We did not detect cardiolipin which has been reported to be present in lobster nerve in only very small amounts[34].

Phosphorus determination

The iodine staining was allowed to fade and the delineated areas scraped off and transferred into test tubes. Phosphorus was determined in presence of the silicic acid. To each test tube, 0.2 ml of conc. H_2SO_4 was added, followed by digestion in a heating block at 240–250° for 30 min. After cooling, 3 drops of 30% H_2O_2 were added and the tubes returned to the heater for another 15 min. Addition of H_2O_2 and heating was repeated when necessary. The color was developed according to BARTLETT[32]. 1 μg P in a 4-ml final volume gave an A reading of about 0.220 in a Beckman Model B spectrophotometer, at 830 mμ.

Action potential measurements

External electrodes were used for measuring the conducted action potential of nerves from the walking legs of lobster. The procedure was similar to that previously described for the squid giant axon[1]. A relatively large stimulating voltage was used in order to get the maximum height of the compound action potential from this multifibered preparation. The electrical response of the preparation was checked every 5 or 10 min. Axons were exposed to solutions of venoms for 30 min under conditions identical to those described previously for venom treatment prior to extraction of phospholipids. To test whether acetylcholine or curare were rendered active, we applied venom, phospholipase A or direct lytic factor solutions to the axon for 30 min in concentrations which by themselves did not affect the action potential. They were then replaced by fresh sea water for at least 10 min and then $1 \cdot 10^{-3}$ M acetylcholine or $5 \cdot 10^{-3}$ M curare was added for 30 min unless block of electrical activity was obtained sooner. Reversibility was checked by adding fresh sea water. These concentrations of acetylcholine and curare have no effect on control axons. All solutions were adjusted to a pH between 7.5 and 8.0.

RESULTS

I. Phospholipids in lobster axons

A map of the phospholipids in lobster axons separated by two-dimensional thin-layer chromatography is illustrated in Fig. 1 and their per cent distribution is

presented in Table I. The lysolecithin spot seen in Fig. 1 appeared in only 2 out of the 7 experiments using intact axons (Table I). No cardiolipin spot was found in lobster nerve extract. The intact axons (Table I) were extracted either immediately after removal of the axons from the lobster or kept for 30 min in sea water in order to resemble the conditions used during venom treatment. Since the values obtained by the two procedures did not significantly differ, they were all included in the means listed under Intact in Table I. The supernatants of intact tissue incubated with sea water for 30 min did not contain significant amounts of solubilized phospholipid. The axons homogenized in sea water were kept for 30 min prior to extraction with chloroform and methanol. Slight splitting of lecithin and phosphatidyl ethanolamine was seen in the extracts of homogenized control axons, as evidenced by the in-

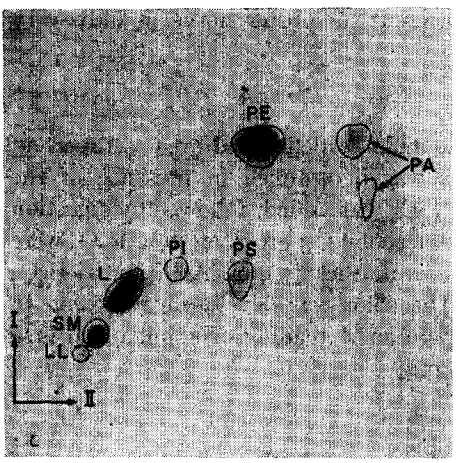

Fig. 1. Chromatographic pattern of the phospholipids in the lobster axon. Solvent systems I and II as described in METHODS. Abbreviations: PE, phosphatidyl ethanolamine; PS; phosphatidyl serine; PI, phosphatidyl inositol; SM, sphingomyelin; L, lecithin; LL, lysolecithin; and PA, phosphatidic acid. The spots were revealed by iodine vapors.

creased values for lysolecithin and phosphatidyl inositol spots (the latter being a combination of phosphatidyl inositol *plus* lysophosphatidyl ethanolamine, see METHODS). Although the level of splitting did not increase with prolonging the incubation time up to two hours, the possible presence of an endogenous phospholipase A cannot be ruled out. Phospholipase A activity and lysolecithin have been found in brain and other tissues[33].

While this work was in progress, data on the phospholipid composition of the lobster leg nerve were published by SHELTAWY AND DAWSON[34]. Our results are in good agreement with these if appropriate corrections are made to account for the different methods used. In our system of thin-layer chromatography the diacyl phospholipids are not separated from the corresponding plasmalogens and alkyl ethers. The values for lecithin and phosphatidyl ethanolamine reported by us represent, each one, the sum of the three analogues and is in good agreement with the sum reported by SHELTAWY AND DAWSON. Our higher values for phospatidyl inositol may be due to the known rapid breakdown of polyphosphoinositides[35] which probably occurred in our procedures so that our results for phosphatidyl inositol should be

compared to the total reported by SHELTAWY AND DAWSON for poly- and monophosphoinositides.

The total lipid phosphorus of the lobster axon was 0.5 ± 0.09 μg P per mg wet weight (19 experiments). This is also in excellent agreement with values reported by SHELTAWY AND DAWSON.

II. Phospholipid splitting and block of electrical activity induced by venom and venom fractions

The phospholipase A activities of venom and venom fractions have been determined and correlated with their effects on electrical activity. The phospholipase

TABLE I

PHOSPHOLIPID DISTRIBUTION IN LOBSTER AXONS

All individual phospholipids are expressed as percentages of the total lipid phosphorus recovered. Mean values ± S.E. are shown for seven experiments with intact axons and three with homogenized axons. The intact axons were directly extracted with chloroform–methanol, the others were first homogenized in sea water and kept for 30 min at room temperature prior to extraction (see METHODS).

Phospholipids	*Intact axons (%)*	*Homogenized axons*
Phosphatidic acid	6 ± 1	6 ± 0.5
Phosphatidyl ethanolamine	31 ± 1	30 ± 1
Phosphatidyl serine	11 ± 1	9 ± 0
Phosphatidyl inositol	2 ± 0.3	5 ± 0.4
Lecithin	37 ± 0.4	35 ± 1
Lysolecithin	0.1 ± 0.01	4 ± 0
Sphingomyelin	13 ± 0.4	11 ± 0

A activities were measured by titrating liberation of free fatty acids from a readily available phospholipid substrate, *i.e.*, egg yolk. The enzymatic activities were in the following approximate relative ratios: Phospholipase A fraction from ringhals venom 100; ringhals venom 30; moccasin venom, 10; *V. palestinae* venom, 2; and rattlesnake venom, 0.7. The direct lytic factor preparation was devoid of phospholipase A activity.

Treatment of lobster axons with moccasin and ringhals venom and phospholipase A in concentrations as low as 1.0, 0.25 and 0.15 mg/ml respectively caused a block of electrical activity in about 30 min which was associated with marked splitting of phosphatidyl ethanolamine, phosphatidyl serine, lecithin and phosphatidic acid (Table II). Lower concentrations did not block electrical activity (Table III). These preparations showed the same order of potency in blocking electrical activity of lobster axons, in splitting of phospholipids in lobster axons, and in their phospholipase A activities as measured on egg yolk.

The degree of phospholipid splitting associated with block of the action potential by the lowest effective concentrations of the active treatments ranged from 34–54% for phosphatidyl ethanolamine, 21–33% for phosphatidyl serine, 30–50% for lecithin and 35–59% for phosphatidic acid. The typical chromatographic patterns of phospholipids in axons in which electrical activity was blocked is shown in Fig. 2. At equal venom concentrations, the per cent phospholipid splitting was significantly

TABLE II

PHOSPHOLIPID SPLITTING AND BLOCK OF ELECTRICAL ACTIVITY IN LOBSTER AXONS

Phospholipid analysis was performed after a 30-min exposure of the axons to treatment. The effects on the action potential were observed in 20–30 min. Number of measurements of action potential are indicated in parentheses. All of the phospholipid values are single determinations. Abbreviations as in Fig. 1.

Treatment	*Concn. (mg/ml)*	*% decrease in action potential*	*% phospholipid splitting*							
			Intact axons				*Homogenized axons*			
			PE	*PS*	*L*	*PA*	*PE*	*PS*	*L*	*PA*
Moccasin venom	3.0		—	41	25	100	100	100	97	100
	1.0	87 ± 9 (3)	34	29	30	59	93	66	90	88
Ringhals venom	3.0	100 ± 0 (2)	90	79	88	72				
	0.5	100 ± 0 (2)	51	35	38	75				
	0.25	100 ± 0 (3)	48	33	46	52	96	85	85	47
Phospholipase A	0.85	100 (1)	59	27	56	69	95	100	81	100
	0.15	67 ± 10 (3)	54	21	50	35				
Direct lytic factor	1.3	100 ± 0 (3)	15	4	6	0				

greater in homogenized than in intact axons. A concentration of direct lytic factor which blocked electrical activity had no significant effect on the phospholipids, in agreement with its lack of phospholipase A activity.

It is of interest that even after marked phospholipid splitting the majority of the lyso products remained in the axon and were not solubilized. A maximum of 6% of the total phospholipid was present in the supernatant when phospholipid splitting was extensive, and consisted mainly of lysophosphatidyl ethanolamine and lysolecithin.

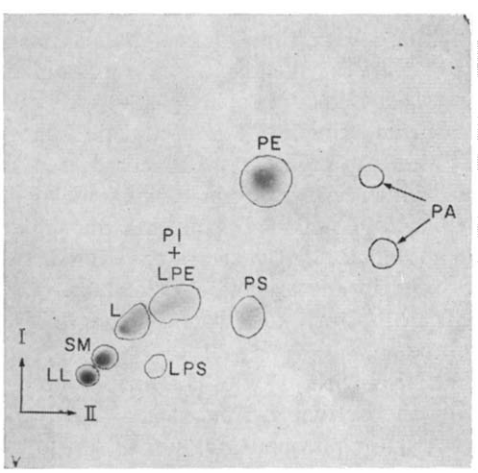

Fig. 2. Chromatographic patterns of axonal phospholipid splitting associated with block of conduction by snake venom. Intact axons were incubated with a 1 mg/ml solution of cottonmouth moccasin venom (see MATERIALS AND METHODS). Electrical activity was blocked. LPE, lysophosphatidyl ethanolamine; LPS, lysophosphatidyl serine. Other abbreviations as in Fig. 1.

III. Phospholipid splitting and effects on axonal permeability induced by venom and venom fractions

Earlier studies have indicated that the inability of acetylcholine and curare to affect conduction is due to the presence of permeability barriers which can be decreased by moccasin venom[2,4,8]. It was therefore of interest to correlate the effects of various venoms and venom fractions on permeability of lobster axons with their effects on axonal phospholipids (Table III). Relatively low amounts of the phospholipase A preparation, ringhals and moccasin venoms rendered the axons sensitive to concentrations of acetylcholine and curare which are normally inactive, indicating that the permeability barrier surrounding the axonal membranes was

TABLE III

PHOSPHOLIPID SPLITTING AND POTENTIATION OF THE EFFECT OF CURARE AND ACETYLCHOLINE ON AXONAL CONDUCTION

Phospholipid analysis was performed after a 30-min exposure of the axons to treatments. The effects on the action potential were observed in 20–30 min. Number of measurements of action potential are indicated in parentheses. All of the phospholipid values are single determinations. Curare and acetylcholine were applied in concentrations of $5 \cdot 10^{-3}$ and $1 \cdot 10^{-3}$ M respectively. Abbreviations as in Fig. 1.

Treatment	*Concn. (mg/ml)*	*% decrease in action potential by*			*% phospholipid splitting*							
		Treatment	*Curare*	*Acetylcholine*	*Intact axons*				*Homogenized axons*			
					PE	*PS*	*L*	*PA*	*PE*	*PS*	*L*	*PA*
V. palestinae venom	12.5	23 ± 14 (3)		10 (1)	22	29	15	61				
	5.0				19	22	15	0	86	100	100	67
	3.0	0 (1)		0 (1)								
Rattlesnake venom	50	0 (1)		10 (1)	8	35	12	0	66	89	72	49
	12.5	16 ± 7 (7)	20 ± 0 (2)	25 ± 5 (2)	8	15	10	—				
Cottonmouth moccasin venom	0.3	0 ± 0 (6)	50 ± 10 (2)	75 ± 25 (2)	20	20	10	—				
Ringhals venom	0.1	18 ± 9 (10)	48 ± 6 (4)	50 ± 4 (4)	24	0	5	0				
Phospholipase A	0.06	13 ± 4 (4)		85 ± 15 (3)	32	8	—	0				
Direct lytic factor	0.6	25 ± 9 (14)		24 ± 4 (5)								

reduced and permitted the compounds to act on the excitable membrane. The degree of phospholipid splitting associated with this increase in permeability (Table III) was less than that observed after block of conduction with these same agents. In contrast, viper and rattlesnake venoms did not significantly affect conduction nor render the axons sensitive to curare or acetylcholine (Table III), even when used in concentrations at which their phospholipase A activities were higher than in concentrations of ringhals venom, moccasin venom or phospholipase A which blocked conduction (Table II). Also the extent of phospholipid splitting in the axons by 12.5 mg/ml viper and 50 mg/ml rattlesnake venoms (Table III) was less than that produced by 0.15 mg/ml phospholipase A, 0.25 mg/ml ringhals or 1.0 mg/ml moccasin venoms (Table II), even though, as mentioned previously, the phospholipase A

activities of these concentrations of viper and rattlesnake venoms are greater than that of the other preparations. There was, however, no significant difference in phospholipid splitting produced by concentrations of moccasin and ringhals venom or phospholipase A which rendered the axons sensitive to curare and acetylcholine, and that induced by rattlesnake and viper venoms which in all concentrations tested were unable to do so. Both *V. palestinae* and rattlesnake venoms induced marked hydrolysis of phospholipids in homogenized axons (Table III). Similarly, by raising the temperature to 37° and prolonging the incubation time to 2 h, 2.5 mg/ml viper venom induced a high degree of phospholipid hydrolysis in non-homogenized axons: 50% of the phosphatidyl ethanolamine, 85% of the phosphatidyl serine, 70% of the lecithin and 87% of the phosphatidic acid. These results are not listed in the table.

IV. Effects of direct lytic factor

Previous studies have shown that the direct lytic factor contained in direct lytic venoms enables venom phospholipases to hydrolyze the phospholipids in red blood cells, platelets and rat-liver mitochondria[16–19]. We thought it to be of interest to find out whether addition of direct lytic factor potentiates the action of viper and rattlesnake venoms on lobster axons, or enhances the effects of low amounts of phospholipase A preparations. Direct lytic factor in concentrations of 0.25–1.2 mg/ml was added to 5 mg/ml of viper or rattlesnake venoms or to 0.1 mg/ml phospholipase A. The splitting of axonal phospholipids by these treatments was not greater than that produced by the equivalent concentrations of venom or phospholipase A alone. The combination with direct lytic factor had also no effect on axonal conduction. No potentiation of phospholipid splitting by direct lytic factor was observed even when combined with viper venom and incubated 2 h at 37°.

DISCUSSION

Previous investigations indicated that phospholipase A is the factor in snake venoms responsible for block of axonal conduction and increased penetration of curare and acetylcholine in the squid giant axon[2,5]. However, since the effects of various snake venoms did not correlate well with their phospholipase activity[5] and since other toxic venom fractions have been described[36], more direct evidence was needed. The present data show unequivocally that block of conduction and increased penetration in lobster axons induced by venoms is due to their phospholipase A fraction. This conclusion is based on two findings: first, loss of excitability and facilitated penetration induced by venoms are each associated with a certain degree of phospholipid splitting in the axons; secondly, a phospholipase A fraction, separated from ringhals venom reproduced, in all respects, the effects of whole venoms. The phospholipase A fraction is devoid of the basic toxins described by MELDRUM[36].

It cannot be ascertained whether the block of excitability or increased penetration induced by venom phospholipase A is associated with the splitting of any particular phospholipid, as the hydrolysis involved all the phospholipid substrates, *i.e.*, phosphatidic acid, phosphatidyl ethanolamine, phosphatidyl serine, and lecithin. Two other phospholipids, sphingomyelin and phosphatidyl inositol, are not substrates of phospholipase A and therefore cannot be involved in the effects produced

by venom or phospholipase A. However, even if the percent hydrolysis is about equal for the four phospholipids that are substrates of phospholipase A, the main sources of lyso products still are phosphatidyl ethanolamine and lecithin which together represent the bulk of axonal phospholipids (68% of the total). The action on these two phospholipids, or one of them, may then be responsible for the functional effects observed.

Splitting of phospholipids by phospholipase A is associated with the liberation of free fatty acids and lysophospholipids, both of which are known to have detergent properties. We have not determined in the lobster whether block of conduction and increased penetration is due to splitting of phospholipids *per se* or due to the subsequent liberation of lytic products, although some observations on the squid axon suggest that the effects of liberated products may be of major importance[37].

The correlation between phospholipid hydrolysis and block of axonal conduction is further confirmed by the results obtained with viper and rattlesnake venoms. These venoms failed to block conduction even when tested in amounts having phospholipase A activity, as tested on egg yolk, greater than that of ringhals and moccasin venom in concentrations which blocked conduction. Phospholipid analysis of axons, however, showed a very low degree of hydrolysis, below the average level associated with block of conduction. Such results emphasize the fact that tests for phospholipase activity, using purified phospholipids or lipoproteins as substrate gives inadequate information about the extent of phospholipid splitting to be expected when phospholipase acts on phospholipids in biological preparations. In contrast to their weak effect on intact axons, both rattlesnake and viper venoms induced a high degree of phospholipid splitting on homogenized preparations, while ringhals and moccasin phospholipase act on both homogenized and intact preparations. These findings further support the concept that phospholipase A from various sources differ in their ability to hydrolyze phospholipids in biological material, as has been found previously on red cells, brain slices and other tissues[16–19,38].

More information is required to interpret the exact significance of the failure of viper and rattlesnake venoms to reduce the permeability barriers. When applied in very high concentration, the extent of phospholipid hydrolysis was similar to that found after those venom treatments which induced axonal sensitivity to curare and acetylcholine. However, we do not know the site of the permeability barrier, nor the regions where phospholipids are being split by viper and rattlesnake venoms. In contrast, block of conduction can be ascribed to an action on the axolemma of the nerve membrane. In the squid axon the Schwann cell seems to be the major permeability barrier[39]. An additional factor to consider is that the nerve of the lobster walking leg is composed of about 800 axons[40]; the poorly penetrating venoms like viper and rattlesnake might only hydrolyze the phospholipids of the peripherally situated axons. Measurements of phospholipid hydrolysis would only then determine an average of those axons with extensive splitting and those with little or none. The electrical response would not be greatly changed since the axons situated in the interior of the nerve bundle may not have been reached by the venoms and may not have had their permeability properties altered. In contrast, ringhals and moccasin venom, which appear to penetrate better than viper and rattlesnake venoms may affect the axons of the bundle more uniformly. It is also possible that the differential effects of the venom on membrane permeability may be correlated with their relative

abilities to hydrolyze plasmalogen *versus* acyl analogues of phospholipids. It was reported, using partially purified substrates, that Cobra and rattlesnake venoms split plasmalogen and acyl forms of phospholipids at different rates[41]. Considerable amounts of phosphatidyl ethanolamine in nerves are known to exist in the plasmalogen form.

Besides phospholipase A another Cobra venom fraction has been shown to play a role in the process of membrane-bound phospholipid splitting. This fraction, the direct lytic factor, is a strongly basic protein which allows hydrolysis by phospholipase A of the "insulated" phospholipids in the membrane of red cells, platelets and mitochondria[16–19]. In the present investigation, no synergistic effect of the direct lytic factor and phospholipase A was found. Whether this different response arises from the specificity of crustacean nerve or from the differences in experimental conditions—artificial sea water rich in Ca^{2+} and Mg^{2+} was used throughout the work—is now under investigation.

Although failing to enhance phospholipase A action, direct lytic factor at concentrations above 1 mg/ml, induced a complete block of the action potential in lobster axons. This effect is different from that induced by phospholipase A, since it is not caused by an enzymatic attack on the axonal phospholipids. It is also evident, in view of the relatively high concentrations of direct lytic factor required to block excitability, that this factor does not play a major role in the block by whole ringhals venom. Basic proteins similar to direct lytic factor, isolated from Hymenoptera[42] and Cobra venom[36] have marked effects on junctional transmission, on muscle membrane and on contraction.

The findings on squid giant axons and the comparison with the results on lobster axons shall be presented in a publication to follow.

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