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

II. STUDY ON SQUID AXONS

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SUMMARY

1. Previous investigations have shown that snake venoms applied in relatively low concentrations to preparations of squid giant axons containing adhering small nerve fibres, allow curare and acetylcholine to reversibly block conduction of the giant axon. Associated with this venom action is a marked increase in membranal permeability. Higher concentrations of venom directly and irreversibly block conduction of the squid giant axon. In contrast when applied to giant axons free of adhering nerve fibres the venoms are devoid of above effects.

In the present investigation the effects of venoms and venom fractions have been tested on both types of preparation, and the phospholipid composition of whole axons, 'envelopes' and extruded axoplasm have been determined.

2. These venom effects appear to be due to its phospholipase A component since an isolated phospholipase A fraction was able to reproduce all the venom effects in preparations of giant axons with fibres and like the venom was inert on giant axons free of adhering fibres. Moreover, the block of electrical activity by venom and phospholipase A and the sensitization of axons to curare and acetylcholine were each associated with a certain level of phospholipid hydrolysis. However, the extent of hydrolysis was similar in preparations with or without adhering fibres.

3. The above results and other findings indicate that lysophosphatides evolved as a result of phospholipase A action may be of greater importance in the course of events following venom treatment than phospholipid splitting *per se*.

INTRODUCTION

Previous studies have shown that certain snake venoms, such as cottonmouth moccasin, facilitate at low concentrations the penetration of acetylcholine and (+)-tubocurarine (curare) into the axons of the walking leg of lobster and the giant axon

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of squid, whereas at higher concentrations they induce an irreversible block of conduction[1–9]. In contrast other venoms, *e.g.* Eastern diamondback rattlesnake venom, are inactive. In a study on lobster axons in which changes induced by snake venoms were correlated with the changes in axonal phospholipids, evidence was found for phospholipase A being the factor in the active venoms responsible for these effects[10].

In the present investigation the studies on the axonal phospholipids following venom-induced block of conduction or increased permeability were extended to the squid axon. This preparation being a single giant fibre has several obvious advantages over the axons from the walking leg of the lobster, which is made up of a bundle of axons, one of the most important being the possibility of determining the action of venom phospholipase A on separated envelopes and axoplasm. Moreover, it was reported that squid giant axons free from adherent small fibres are much less affected by venom than those surrounded by adherent small nerve fibres, with regard to both block of conduction[2] and increased permeability to acetylcholine[6]. These observations suggest that in addition to the phospholipid splitting *per se*, the amount of evolving lysophosphatides, supposedly greater in giant axons with adhering small fibres, might be of importance in the causation of the above effects. A direct evaluation of the phospholipid changes in the two preparations was necessary for the interpretation of these data.

We compared in the present study the degree of phospholipid splitting induced by venom and venom fractions in giant axons free of fibres and in giant axons surrounded by adhering nerve fibres, as well as on separated axoplasm and envelopes.

MATERIALS AND METHODS

Venoms and chemicals

Viper (*Vipera palestinae*) venom was obtained through the courtesy of Dr. E. KOCHWA (Dept. of Zoology of the Tel Aviv University). Ringhals (*Hemachatus 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, sphingomyelin and lysolecithin were a gift of Dr. M. M. RAPPORT from the Albert Einstein College of Medicine, New York, N.Y. Phosphatidic acid (sodium salt) was purchased from General Biochemicals, Chagrin Falls, Ohio, and phosphatidyl ethanolamine from Applied Science Laboratory, State College, Pa. Lysolecithin, prepared from egg lecithin, was purchased from Sigma Chemical Co., St. Louis, Mo. Fiske–SubbaRow reagent was purchased from Fisher Scientific Co. [*N-Me*-^{14}C] Acetylcholine chloride was purchased from New England Nuclear Corp.

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[11]. Heat treatment, dialysis of the phospholipase A fraction, and measurement of catalytic rates on egg-yolk phospholipids were carried out as described in a previous communication[10]. Protein content in venoms and the separated venom fractions were determined according to the method of LOWRY *et al.*[12].

Dissection of axons and extrusion of axoplasm

The giant axons of the squid, *Loligo pealii*, were dissected as previously described[1,2], *i.e.* the majority but not all of the small adhering nerve fibres were removed. We will refer to these preparations as 'giant axons with fibres'. A number of axons were further dissected, removing all the small fibres, and these preparations will be referred to as 'giant axons'.

In a number of experiments the axoplasm was extruded both from the giant axon and from the giant axons with fibres. The remainder after extrusion of axoplasm shall be called 'envelope'. The axoplasm was extruded by means of a tygon-coated roller, and the axoplasmic droplets forming at the free end of the axon were collected by suction into a pre-weighed capillary[3]. By this procedure, the adhering nerve fibres having a very small diameter would contribute very little, if at all, to the axoplasm extruded from the giant axon.

Venom treatment and recording of electrical activity of the squid giant axon

The axons having both ends ligated with threads were placed in chambers and covered with filtered natural sea water buffered with 1 mM Tris to pH 7.7–8.1, and containing the venom or venom fractions. After a 30-min incubation at a temperature of 16–20°, the venom solution was removed and the axons thoroughly washed by circulating filtered sea water through the chamber. Controls were run in the same conditions, only the venom was omitted. Electrical activity of the axons was recorded either with extracellular or intracellular electrodes as previously described[1,2]. Small nerve fibres do not respond to the minimal voltage required to stimulate the giant axon. All electrical recordings are therefore only giant axon responses. Some axons were then used for lipid extraction while others were exposed to acetylcholine or curare in order to determine whether the axons had been rendered sensitive to these agents by the venom treatment. In control axons acetylcholine and curare do not affect conduction[1–7].

Extraction and chromatography of lipids

The axons were blotted on filter paper and rapidly weighed. 6–8 giant axons with fibres having a total weight of 100–150 mg, or 10–15 giant axons having a total weight of 20–30 mg were pooled for each determination. 5–10 mg of envelope from the giant axon and 30–100 mg of axoplasm were used for each lipid extraction. Lipids were extracted by homogenization of whole axons and separated envelope in chloroform–methanol (2:1, v/v) in a glass homogenizer as described by FOLCH, LEES AND SLOANE-STANLEY[13]. Glass capillaries containing axoplasm were crushed in chloroform–methanol and extracted as above. The extracts were washed with 0.04 % $CaCl_2$ (ref. 13), evaporated to dryness and reextracted in chloroform–methanol, the final volume being brought to 1 ml. This extract or aliquots thereof was either applied to thin-layer chromatographic plates (see below) for determination of individual phospholipids, or directly analyzed for total lipid phosphorus values (see Table I) by the method of BARTLETT[14], following digestion with H_2SO_4 and H_2O_2.

Two-dimensional thin-layer chromatography was carried out as described previously[10]. From the extracts of giant axons with adhering fibres and their envelopes, duplicate plates were run, applying aliquots that contained approx. 10 μg phosphorus. However, for extracts of the giant axon, its envelope and all studies with axoplasm,

TABLE I

PHOSPHOLIPIDS IN SQUID AXONS

All individual phospholipid values are expressed as percentages of the total lipid phosphorus recovered. Results are recorded as means ± S.E. PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol; L, lecithin; SM, sphingomyelin.

Preparation	*Total lipid P*		*Distribution* (%)					*Number of experiments*
	μg/mg wet wt.	*Number of experiments*	*PE*	*PS*	*PI*	*L*	*SM*	
Giant axon with fibres	0.38 ± 0.02	10	27 ± 1	7 ± 0.3	2 ± 0.3	52 ± 1	12 ± 1	4
Giant axon	0.23 ± 0.04	2	31 ± 1	8 ± 0.3	5 ± 0.2	45 ± 1	11 ± 0.2	2
Envelope of giant axon	—*	3	28 ± 1	11 ± 1	5 ± 0.4	40 ± 2	16 ± 1	2
Axoplasm	0.09 ± 0.01	3	31 ± 2	7 ± 1	7 ± 1	56 ± 1	0	2

* Because of varying degrees of drying of envelopes during and following extrusion of axoplasm reliable wet weight measurements could not be made, giving rise therefore to erratic total lipid P/weight ratios (range 0.5–2.0 μg/mg).

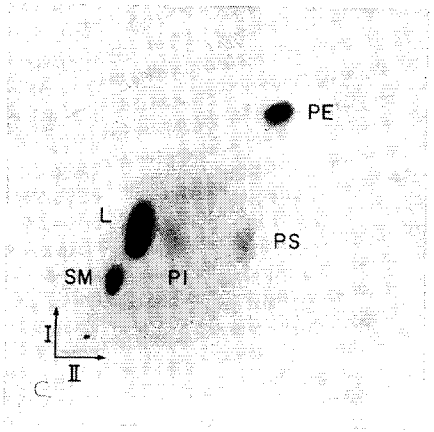

Fig. 1. Chromatographic pattern of the phospholipids in the squid giant axon. Solvent system I: chloroform–methanol–water (65:25:4, by vol.); solvent system II: 3-heptanone–acetic acid–water (80:50:10, by vol.). PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol; L, lecithin; SM, sphingomyelin. Spots revealed by iodine vapour.

all the lipid extract had to be applied to a single plate as it contained no more than 3–9 μg P. After the chromatographic run, the spots were revealed with iodine vapour and encircled. For identification we compared the spots with known markers, and carried out specific colour reactions as previously described[10]. Phospholipid phosphorus in the separated spots was assayed as previously described[10] using the method of BARTLETT[14].

Penetration of [N-Me-^{14}C]acetylcholine into the axoplasm

Following exposure of axons to venom solutions or to normal sea water for 30 min the axons were rinsed and placed for 1 h in a solution containing [^{14}C]acetylcholine bromide *plus* non-radioactive acetylcholine carrier to give a final concentration of $4.5 \cdot 10^{-3}$ M. The solution also contained $2.4 \cdot 10^{-4}$ M non-radioactive physostigmine salicylate in order to decrease enzymatic hydrolysis. The radioactivity of the solution was about $8 \cdot 10^{5}$ disint./min per ml. The percent penetration of acetylcholine into the axoplasm was calculated by comparing the disintegration values expected from equilibrium with the actual radioactivity found in extruded axoplasm, as described in detail in previous papers[3,6].

RESULTS

I. Phospholipid distribution in squid axons

The following phospholipids were tentatively identified in the extracts of the squid giant axon with or without adhering nerve fibres: phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, lecithin and sphingomyelin. No phosphatidic acid was detected. In our system of thin-layer chromatography the diacyl phospholipids are not separated from the corresponding plasmalogens and alkyl ethers. The values for phospholipids reported by us represent, each one, the sum of the three analogues. The pattern of the phospholipids separated by thin-layer chromatography is illustrated in Fig. 1, and their percent distribution listed in Table I. Under the column headed *Number of experiments* are listed the number of different lipid extracts which were analyzed. In some cases duplicate runs were carried out on the

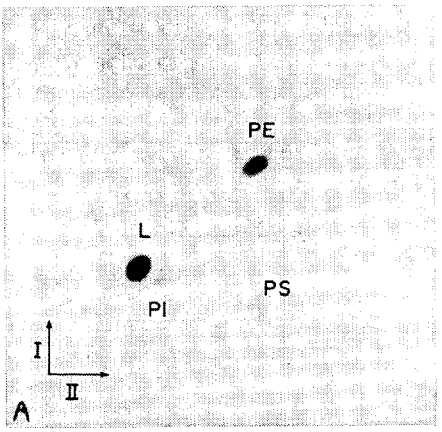

Fig. 2. Chromatographic pattern of the phospholipids in extruded axoplasm. Abbreviations as in Fig. 1.

same lipid extract (see METHODS), however, this is considered only a single experiment. The phospholipid concentration and percent distribution in giant axons in which the small adhering fibres were removed by careful dissection differ somewhat from that in preparations of giant axon *plus* adhering fibres (Table I).

As expected, separate analysis of axoplasm, known to constitute 80 % of the total weight of the giant axon[15] revealed a much lower phospholipid concentration than the giant axon with or without fibres. It is noteworthy that the axoplasm contains all the phospholipids found in the envelope with the exception of sphingomyelin (Fig. 2, Table I). In addition to the experiment shown in Table I, the absence of sphingomyelin was confirmed in all subsequent analyses of axoplasm (Figs. 6 and 7). This typical feature was taken as an index for careful extrusion of the axoplasm, since sphingomyelin was found to appear only after obvious contamination of the axoplasm with envelope material.

II. Effects of venoms, phospholipase A and the direct lytic factor on giant axons with adhering nerve fibres

It was reported in a previous paper that 50 μg moccasin venom per ml of sea water blocks irreversibly the electrical activity of the squid giant axon provided the preparation contained adhering nerve fibres. When used in a lower concentration which did not block the action potential, it rendered the axons sensitive to curare and acetylcholine[2,4]. Moccasin venom when tested in this study gave essentially the same results as reported before, *i.e.* blocked electrical activity in a concentration of

TABLE II

EFFECTS OF VENOMS AND VENOM FRACTIONS ON THE ACTION POTENTIAL OF THE SQUID GIANT AXON

Giant axons with fibres were exposed to venom or venom fractions for 30 min, except for those treatments which blocked conduction (15–30 min). All effects on the action potential were irreversible. The action potential was measured with extracellular electrodes. Results are recorded as means ± S.E.

Venom or venom fraction	*Concn. (μg/ml)*	*Number of experiments*	*Decrease action potential (%)*
Phospholipase A	10	12	6 ± 3
	15	4	33 ± 10
	25	7	47 ± 17
	50	7	100 ± 0
Ringhals	25	2	30 ± 0
	50	3	100 ± 0
V. palestinae	200	2	10 ± 5
	400	2	100 ± 0
Rattlesnake	1000	9	10 ± 5
	4000	2	100 ± 0
Direct lytic factor	200	4	9 ± 2
	500	9	47 ± 11*
	1400	2	100 ± 0

* In three of the experiments, with two different batches of direct lytic factor, a block of axonal conduction was observed.

TABLE III

EFFECTS OF ACETYLCHOLINE AND CURARE ON THE ACTION POTENTIAL OF SQUID GIANT AXON FOLLOWING ITS PRETREATMENT WITH VENOM OR VENOM FRACTIONS

The preparation used consisted of the squid giant axon with fibres. All pretreatments in the concentration listed below, caused a decrease in the action potential of 30% or less in 30 min. The action potential was recorded with extracellular electrodes. Results given as mean ± S.E.

Pretreatment	*Concn.* (*μg/ml*)	*Curare* (*M*)	*Acetylcholine* (*M*)	*Number of experiments*	*Exposure* (*min*)	*Decrease action potential* (%)	*Reversibility* (%)
Phospholipase A	10	$1.4 \cdot 10^{-3}$	—	3	10	90 ± 11	30
	10	$1.4 \cdot 10^{-4}$	—	4	20	85 ± 15	20
	10	$1.4 \cdot 10^{-5}$	—	2	30	0 ± 0	—
	15	—	$4.5 \cdot 10^{-3}$	4	10–30	100 ± 0	—
Ringhals	15	$1.4 \cdot 10^{-3}$	—	2	30	75 ± 0	90
V. palestinae	100	$1.4 \cdot 10^{-3}$	—	2	30	80 ± 10	30
Rattlesnake	1000	$1.4 \cdot 10^{-3}$	—	8	20–30	65 ± 13	60–100
Direct lytic factor	500	$1.4 \cdot 10^{-3}$	—	3	30	0 ± 0	—
	500	—	$4.5 \cdot 10^{-3}$	2	30	15 ± 15	—

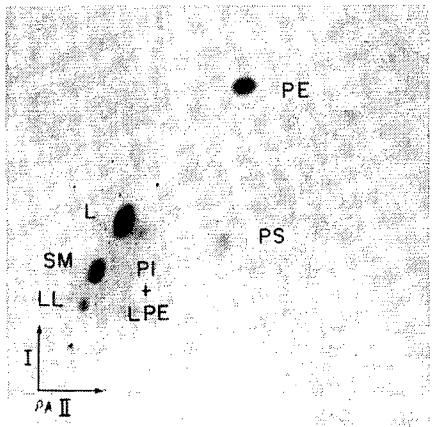

Fig. 3. Phospholipid splitting in squid giant axons with fibres at the time of block of conduction by phospholipase A. The axons were incubated in a 50 μg/ml solution of phospholipase A for 30 min. LPE, lysophosphatidyl ethanolamine; LL, lysolecithin; other abbreviations as in Fig. 1.

50 μg/ml, and in 15 μg/ml made possible the effect of acetylcholine and curare on the excitable membrane. In contrast rattlesnake venom had previously been found to be inactive on the squid giant axon in concentrations as high as 1 or 2 mg/ml. A new batch of rattlesnake venom used in the present study did block electrical activity; however, a concentration of 4 mg/ml was required (Table II). Ringhals and *V. palestinae* venoms, which have not previously been tested on the squid axon, also blocked conduction (Table II). Amounts of rattlesnake, vipera and ringhals venoms which did not block conduction, rendered the axons sensitive to curare (Table III). An isolated phospholipase A fraction from ringhals venom was active both in inducing block of electrical activity (Table II) and as pretreatment following which acetylcholine and curare became effective (Table III).

Phospholipid analyses were performed on the axons treated with amounts of venom or venom fractions which either blocked conduction or sensitized the axons to curare and acetylcholine. The concentrations of phospholipase A and of whole ringhals, moccasin, viper and rattlesnake venoms which blocked conduction also hydrolyzed the three phospholipid substrates in squid axons (Table IV and Fig. 3). For each individual treatment, at least two of the substrates were split, one of them being consistently lecithin, which also represents the highest percentage (52 %) of axonal phospholipid. The hydrolysis of lecithin ranged between 30 and 48 % of the total with the various treatments, while that of phosphatidyl ethanolamine varied between 4 and 37 % and that of phosphatidyl serine between 0 and 57 %. All venoms

TABLE IV

SPLITTING OF PHOSPHOLIPIDS BY VENOM AND VENOM FRACTIONS IN GIANT AXON WITH SURROUNDING SMALL NERVE FIBRES

Intact axons were exposed to treatments for 30 min. The percentage splitting was derived from the decrease of phospholipids in venom-treated axons as compared to control axons. These values were in excellent agreement to those obtained by assaying the individual lysophospholipids produced as a result of phospholipase A or venom action. Sphingomyelin and phosphatidyl inositol were not hydrolyzed by venom phospholipase A, and are therefore not recorded in the table. Abbreviations as in Table I.

Venom or venom fraction	*Concn. (μg/ml)*	*% Phospholipid splitting by concentration which:*					
		block action potential			*sensitize axons to curare*		
		PE	*PS*	*L*	*PE*	*PS*	*L*
Phospholipase A	50	37	10	48			
	15				16	0	21
Ringhals	50	16	0	39			
	15				8	10	16
Moccasin	50	31	30	30			
	15				0	10	17
V. palestinae	400	4	0	36			
	100				0	0	23
Rattlesnake	4000	35	57	48			
	1000				0	0	30
Direct lytic factor	500	0	0	10			

and the phospholipase A fraction when used in lower amounts which did not affect conduction but which allowed curare to block conduction also induced phospholipid splitting but to a smaller degree. Under these conditions the lecithin component was consistently hydrolyzed, to an extent of 16–30 %. Phosphatidyl ethanolamine and phosphatidyl serine were split from 0 to 16 and 0 to 10 %, respectively (Table IV).

The direct lytic factor while being devoid of phospholipase A activity[10] blocked conduction in concentrations varying from 500 μg/ml to 1400 μg/ml in the various batches tested (Table II). A batch which did not block conduction in 500 μg/ml was also found inefficient as a pretreatment (Table III). Even a sample of direct lytic factor which blocked conduction in a concentration of 500 μg/ml did not significantly hydrolyse the axonal phospholipids (Table IV). Although the combination of direct lytic factor *plus* phospholipase A has a synergistic action in splitting phospholipids of certain membranes[11,16–18], no such effect was found on the squid axon. As to be expected, therefore, addition of direct lytic factor did not increase the effect of venom or phospholipase A on conduction.

In addition to measuring the effects of direct lytic factor, phospholipase A and ringhals venom on the action potential of the squid giant axon with extracellular electrodes, we also made intracellular recordings. In appropriate concentrations all blocked conduction and markedly reduced the resting potential. Phospholipase A (50 μg/ml) induced a block of conduction which was not associated with any change in resting potential, however, subsequent to this block the membrane became completely depolarized (Fig. 4). Ringhals venom (100 μg/ml) appeared to have a similar effect. In contrast, a much higher concentration of direct lytic factor (1000 μg/ml) blocked conduction only after partial depolarization of the membrane, which did not become complete (Fig. 5).

Since cottonmouth venom pretreatment of the giant axon with fibres is known to increase the penetration of ^{14}C-labelled acetylcholine into the axoplasm it was of

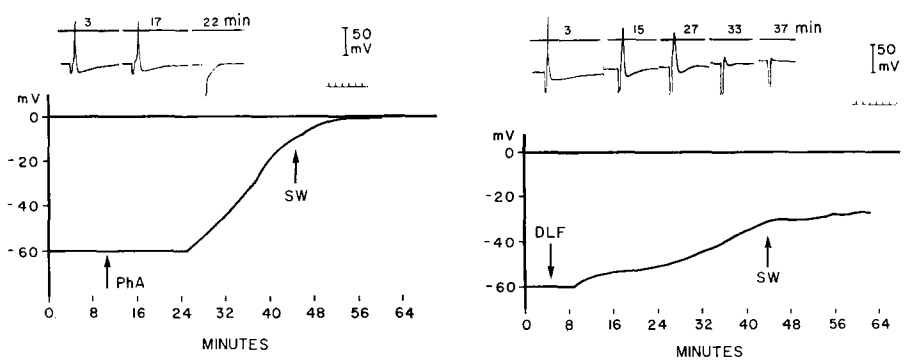

Fig. 4. Effects of phospholipase A on the resting and action potentials of the squid giant axon. Intracellularly recorded action potentials are shown at top of figure, and time course change in resting potential is shown below. Phospholipase A (50 μg/ml) applied at 10 min blocked conduction at 22 min and subsequently depolarized the membrane. Ph A, phospholipase A; SW, sea water. Time signal calibration in msec is shown beneath the calibration for mV. Squid giant axons with fibres were used.

Fig. 5. Effects of direct lytic factor (DLF) on the resting and action potentials of the squid giant axon. Direct lytic factor (1000 μg/ml) applied at 5 min induced a slow depolarization of the membrane leading to block of conduction at 33 min. Other conditions as in Fig. 4.

interest to compare the abilities of other venom fractions, to facilitate penetration. As seen in Table V block of conduction by whole venoms and the phospholipase A fraction is associated with marked increases in the penetration of acetylcholine above the level observed in control axons. Sensitization of axons to acetylcholine by non-blocking amounts of venom is also associated with an increased penetration of acetylcholine, although not as great as observed with amounts of venom and phospholipase A which block conduction. In contradistinction block of conduction by direct lytic factor is associated with a much lower degree of acetylcholine penetration than produced by blocking amounts of venom and phospholipase A. An amount of direct lytic factor which did not affect conduction failed to sensitize the axons to acetylcholine and did not significantly affect acetylcholine penetration.

TABLE V

CORRELATION BETWEEN PENETRATION OF [*N-Me*-^{14}C]ACETYLCHOLINE INTO THE AXOPLASM OF THE SQUID GIANT AXON AND EFFECTS ON CONDUCTION

Giant axons with fibres were pretreated for 30 min, and ability of the giant axon to conduct was monitored. The axons were then exposed for 1 h to $4.5 \cdot 10^{-3}$ M acetylcholine solution having a radioactivity of about $8 \cdot 10^5$ disint./min per ml and the electrical recordings were repeated prior to extrusion of axoplasm. If the axonal membrane were to offer no barrier to free diffusion (100% penetration), the same radioactivity as in the external solution would be expected in the axoplasm, assuming that 1 mg of axoplasm contains 1 μl of solution into which acetylcholine can diffuse. On this basis percent penetrations were calculated. The weight of axoplasm extruded ranged between 2 and 6 mg per axon. + indicates block of conduction; — no effect. All results are given as means ± S.E.

Pretreatment	*Concn. (μg/ml)*	*Number of experiments*	*Penetration of acetylcholine (%)*	*Effects on conduction by pretreatment*	*Effects on conduction by acetylcholine*
None	—	10	3 ± 0.3		—
Phospholipase A	50	2	87 ± 12	+	
Ringhals	15	2	18 ± 5	—	+
	50	1	60	+	
V. palestinae	200	2	8 ± 0	—	+
	400	2	41 ± 5	+	
Rattlesnake	1000	2	11 ± 3	—	+
	4000	4	47 ± 7	+	
Direct lytic factor	100	2	5 ± 1	—	—
	500	3	9 ± 1	+	

III. Effects of phospholipase A on the phospholipids in giant axons free from nerve fibres

It was previously reported[2,6] that amounts of venom which cause a 100% decrease of the action potential and marked increase in penetration of [^{14}C]acetylcholine in axons with adhering fibres were much less effective on preparations in which the small fibres adhering to the giant axon were removed. We have now found that a phospholipase A preparation as well had little effect on the action potential of the giant axons when freed of adhering fibres in contrast to its potent effects on the giant axons with fibres. We therefore examined the phospholipids in giant axons free of adhering fibres and treated with the same amount of phospholipase A which

blocked conduction in the axons with fibres. As seen in Table VI, the degree of splitting of the two major phospholipids, phosphatidylethanolamine and lecithin following venom treatment does not differ markedly from that found in axons having adhering nerve fibres (Table IV).

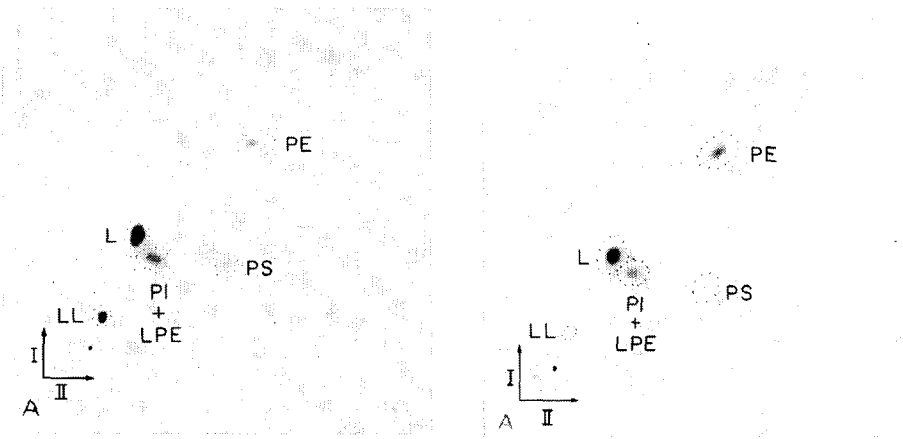

Fig. 6. Phospholipid splitting induced by phospholipase A in the axoplasm of giant axons with fibres. The axons were incubated in a 50 μg/ml phospholipase A solution for 30 min; then the axoplasm was extruded. Abbreviations as in Figs. 1 and 3.

Fig. 7. Phospholipid splitting induced by phospholipase A in the axoplasm of giant axons free of adhering fibres. Conditions and abbreviations as in Fig. 6.

TABLE VI

EFFECTS OF PHOSPHOLIPASE A ON PHOSPHOLIPIDS IN GIANT AXONS, ENVELOPE, AND AXOPLASM

Intact giant axons with and without surrounding small nerve fibres were exposed for 30 min to 50 μg/ml phospholipase A. Conduction was blocked by this treatment only in the giant axons having adhering small fibres. The lipids were separately analyzed in extruded axoplasm and in remaining envelope. Abbreviations as in Table I. The percentage splitting was calculated as described in Table IV. Values for phosphatidyl serine are not recorded since the small amounts of tissue available and its relatively small percent of the total phospholipids rendered the values erratic.

Preparation	*Splitting* (%)	
	PE	*LEC*
Whole giant axons	30	14
	48	38
Giant axons		
Envelopes	73	83
	72	57
Axoplasm	53	17
	57	0
Giant axon with fibres		
Envelopes	76	69
	74	69
Axoplasm	53	31
	47	42

It appeared of interest to separate following venom treatment the axoplasm from the envelopes in preparations with and without adhering small nerve fibres. Once more, the phospholipid hydrolysis in the envelopes of the two preparations was similar and reached high values, averaging 74 % of the phosphatidyl ethanolamine and 70 % of the lecithin. Phospholipid splitting extended also to the axoplasm of both preparations. About 50 % of the phosphatidyl ethanolamine was hydrolysed in both cases but little effect was found on the axoplasmic lecithin of giant axons free of adhering fibres in contrast to that of axons surrounded by attached fibres (Table VI and Figs. 6 and 7).

DISCUSSION

The giant axon of the squid has been extensively used in physiological studies and there is considerable information available on the proteins[19], electrolytes[15,20] and other dialyzable constituents present[21–23]. In contrast, however, the lipid composition has not been analyzed in detail. In earlier studies by McColl and Rossiter[24,25], which were corrected in a personal communication to Geren and Schmitt[26], they reported on the cholesterol and phospholipids in various regions of squid nervous tissue including the giant axon; however, the analytical methods available at the time did not allow a quantitative separation of the individual phospholipids. Data presented in this paper show that the phospholipid distribution in squid axons is similar to that found previously in lobster axons[10], except for an inability to detect phosphatidic acid in the squid axon, which was reported as being present by Larabee and Brinley[27]. Our data confirm the very low phospholipid concentration in axoplasm reported by McColl and Rossiter[24,25]. The presence of filaments and mitochondria in the axoplasm of the squid giant axon[26,28], raise the question whether the axoplasmic phospholipids are associated with the particulate elements or are present as soluble lipoproteins. It is of interest that sphingomyelin, long considered a typical myelin lipid is indeed present in the sheath of the giant axon and absent from the axoplasm.

The present results confirm the finding, previously demonstrated on lobster axons[10] that the venom-induced block of excitability and sensitization of the axons to acetylcholine and curare allowing them to block conduction are due to phospholipid hydrolysis by venom phospholipase A. These effects are associated with a graded level of phospholipid splitting and the action of whole venom is reproduced by an isolated phospholipase A fraction. The hydrolysis of axonal phospholipids by venom phospholipase A also markedly increases membranal permeability as evidenced by increased acetylcholine penetration allowing it to reversibly block conduction. Acetylcholine has no effect on conduction in control axons, and the very low levels of apparent penetration observed might be due to contamination of axoplasm during extrusion or to the presence of lipid-soluble impurities in the radioactive acetylcholine[3].

We previously reported that *V. palestinae* and rattlesnake venoms differ from those of cottonmouth moccasin and ringhals by being unable to block conduction of lobster axons due to their inability to penetrate and hydrolyze phospholipids in this multifibred preparation[10]. In contrast, the above venoms were all active on the squid axon, although relatively large concentrations of rattlesnake and viper venoms were

required, correlating with their low phospholipase A activities[10]. The single giant axon of the squid may offer less of a barrier to the penetration of rattlesnake and viper venoms than the lobster axon.

In our study on lobster axons we reported that the direct lytic factor present in ringhals venom is able to induce block of conduction in relatively high concentration through a mechanism which does not involve phospholipid splitting in contrast to the block by phospholipase A. Also, there was no synergistic effect between the direct lytic factor and the phospholipase A in contrast to findings in red cells and other preparations[11,16–18]. The present data confirm these observations in the squid axon and show in addition that the effect of direct lytic factor on electrical activity differs from that produced by phospholipase A, in respect to the state of the membrane potential at the time of and subsequent to the block of conduction. Moreover, block of conduction by the direct lytic factor is not accompanied by an increased penetration of externally applied acetylcholine into the axoplasm, which occurs after whole venom and phospholipase A treatment.

As found in previous investigations[2,6] and confirmed again in the present study, venom treatment is much less efficient in depressing the action potential and increasing permeability in giant axons free of adhering nerve fibres than in those with small fibres attached. We have now found that the overall degree of phospholipid splitting induced by phospholipase A in the two preparations is similar. However, phospholipid analysis of whole axons gives only little information on the state of the phospholipids in the axolemma, which represents a very small fraction of the tissue analyzed. Indirect information was obtained by separate analyses of the extruded axoplasm of axons with fibres which showed more than twice as much phospholipid splitting as in the extruded axoplasm of the giant axons free of fibres. The main axoplasmic phospholipid-lecithin was only little hydrolyzed in the latter preparation. It seems reasonable to assume, therefore, that the phospholipid hydrolysis in the axolemma, which is immediately adjacent to the axoplasm, is also less marked in the axon free of fibres. This finding would be consistent with the effects observed on electrical activity. The better penetration of phospholipase A into the axoplasm of the giant axon with fibres may be due to the effects of the evolved lysoproducts, *i.e.* lysophospholipids and fatty acids. Obviously, the amounts of lysoproducts produced by enzymatic action in the axons with fibres are much larger than in the giant axons free of surrounding fibres. These lysoproducts with known detergent properties may facilitate the inward spreading of phospholipase A by loosening the axonal structure. Lysophosphatides, in addition to facilitating the action of phospholipase A, may also have direct effects on the membrane. Another detergent, cetyl trimethylammonium chloride has been found to block conduction and render squid giant axons sensitive to curare[1]. Preliminary experiments on the effects of lysophosphatides on the giant axon show that they are able to block conduction and increase permeability. These results to be published at a later date support the view that evolved lysophosphatides may be of major importance in the course of events following venom treatment.

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