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MAINTENANCE OF AXONAL CONDUCTION AND MEMBRANE PERMEABILITY IN PRESENCE OF EXTENSIVE PHOSPHOLIPID SPLITTING*

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Abstract—The importance of phospholipids for the maintenance of axonal conduction and membrane permeability has been investigated by treating squid giant axons with phospholipases, lysosphospholipids and fatty acids.

It was found that phospholipase C, from *Cloistridium welchii*, induces an extensive splitting of axonal phospholipids, without however blocking conduction or increasing the penetration of a lipid-insoluble compound into the axoplasm of squid giant axons. These findings are in sharp contrast with the results obtained with phospholipase A, electrophoretically separated from Ringhals venom, where a close relationship between phospholipid splitting, block of axonal conduction, and increased penetration has been reported. Phospholipase B from bovine pancreas, and D from cabbage, did not markedly hydrolyze the axonal phospholipids nor did they affect conduction or penetration.

Both purified lysolecithin and a mixture of lysophosphatides, prepared by the action of venom phospholipase A on the phospholipids of squid axons, blocked conduction and increased penetration into giant axons. In contrast to phospholipase A, which affects giant axons only if surrounded by adhering small nerve fibers, lysolecithin and the lysophosphatide mixture acted equally well on giant axons with or without adhering small nerve fibers. The other product of phospholipase A action, free fatty acids, was inert on the giant axon.

On the basis of these and earlier findings, it is concluded that extensive splitting of axonal phospholipids by phospholipases is compatible with maintenance of normal function unless the hydrolytic products are themselves active. This conclusion would explain the marked differences in action found between phospholipases A and C.

SEVERAL specific functions of phospholipids in neural conduction have been proposed, such as their possible role in active transport,[1] in genesis of bioelectricity[2] and in interaction with local anesthetics.[3] In addition, a general function of the phospholipids may be for the maintenance of structural and permeability properties of substructures of cells such as the Schwann cell or the plasma membrane. Disruption of phospholipids by venom phospholipase A has been reported to increase permeability in frog atria

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and sciatic nerve,[4, 5] while Tobias has concluded, on the basis of studies with phospholipase and proteolytic enzymes, that membrane resistance and capacitance in the lobster nerve may be linked to phospholipids rather than to proteins.[6]

In an attempt to evaluate the essentiality of phospholipids in axonal conduction and permeability, we have extended our earlier studies on the effects of venom and phospholipase A on axonal membranes.[7–16] By using preparations of squid giant axons containing many adhering small nerve fibers, we have shown that snake venoms block conduction of the giant axon, and, when used in nonblocking amounts, facilitate the penetration of and render the giant axon sensitive to acetylcholine (ACh) and curare.[7–14] Phospholipase A was shown to be the component of the venoms responsible for these effects, since venom action was accompanied by graded axonal phospholipid splitting and since a phospholipase A fraction isolated from venom mimics in all respects the action of whole venom.[15–16] These results seemed therefore to indicate an essential role of phospholipids in maintenance of axonal function and permeability. However, it was also observed that snake venoms and their phospholipase A fraction did not block conduction or facilitate the penetration of ACh into giant axons that had been freed by careful dissection of all the small surrounding nerve fibers.[8, 13, 16] Phospholipid analyses, however, indicated that the extent of phospholipid splitting by venom or phospholipase A was about equal in preparations with or without adhering small nerve fibers.[16] The small nerve fibers surrounding the giant axon may represent a major source of lytic products, such as lysophosphatides and free fatty acids, which arise from the action of phospholipase A on membranal phospholipids. The effects of phospholipase A may therefore have been due not to phospholipid splitting *per se*, but rather to the action of evolved lytic products.

In the present communication, we have attempted to evaluate both the role of products in the action of phospholipase A and whether nerve conduction and normal permeability could be maintained in the presence of extensive phospholipid splitting. We have studied the effects of lysophospholipids and fatty acids on nerve conduction and permeability and compared their effects to those of phospholipase A. We have also compared phospholipid splitting induced by phospholipases B, C and D with their effects on axonal conduction and permeability, since unlike phospholipase A these enzymes give rise to hydrolytic products devoid of detergent or lytic properties.

MATERIALS AND METHODS

Phospholipases and chemicals. Phospholipase B (lysolecithin acyl-hydrolase, EC 3.1.1.5) from bovine pancreas was purchased from Pierce Chemical Co., Rockford, Ill. In order to remove the ammonium sulfate present, the turbid preparation was clarified by dialysis against filtered natural sea water, buffered with 1 mM Tris (hydroxymethyl) aminomethane to pH 7·7 to 8·1. As a source of phospholipase C (phosphatidylcholine cholinephosphoydrolase, EC 3.1.4.3), a partially purified alpha toxin of *Cloistridium welchii* with an activity of 4·5 units/mg was purchased from Mann Research Laboratory, New York, N.Y. The preparation was readily soluble in sea water. The phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) prepared from cabbage with a specific activity of approximately 0·5 unit/mg was supplied by Calbiochem, Los Angeles, Calif. This preparation was not readily soluble in sea water buffered at pH 7·7 to 8·1 or at pH 5·6. Lysolecithin prepared from egg lecithin and containing mainly palmitic and stearic acid was a product of the Sigma Chemical Company, St.

Louis, Mo. Oleic and stearic acids were products of Fisher Scientific Company, N.J., and linoleic acid was obtained from Matheson, Coleman & Bell, Norwood, (Cincinnati) Ohio. Oleic and linoleic acids were emulsified in buffered sea water by sonication. The stearic acid was suspended in sea water in the same manner. Crystallized human serum albumin was purchased from Pentex Inc., Kankakee, Ill. Acetylcholine (*N*-methyl-^{14}C) chloride was purchased from New England Nuclear Corp. Ringhals (*Hemachatus haemachatus*) venom was purchased from Pierce Chemical Company, Rockford, Ill.

Preparation of "lysophosphatides". Two g of giant axons with surrounding nerve fibers was homogenized by hand in Tenbroek tissue grinders and incubated overnight with 10 mg Ringhals venom in 2 ml distilled water at room temperature. Two types of control incubations were also carried out, one without venom and one without axons. In the latter control, 0·1 g egg albumin was used instead of axons, since this is the approximate amount of protein which would be in 2 g (wet wt.) of axons. The lipids were first extracted with methanol:chloroform (3:1, v/v) then evaporated to dryness and reextracted in chloroform:methanol (2:1, v/v) and washed with 0·04% $CaCl_2$ as described by Folch *et al.*[17] One aliquot was used for determination of total lipid phosphorus, while another was applied to thin-layer chromatographic plates giving the pattern illustrated by Fig. 1. Phosphorus content was assayed by the method of Bartlett.[18] The criteria used for identifying the spots has been previously described.[15, 16] The mixture of phospholipids consisted of: lysolecithin (LL), 50%; lysophosphatidyl ethanolamine (LPE), 25%; lysophosphatidyl serine (LPS), 5%; phosphatidyl inositol (PI), 2%; sphingomyelin (SM), 13% and material left at the origin amounting to 5% of the total phosphorus. The organic solvents were evaporated from the remainder of the extract and the entire residue was suspended in buffered sea water and cleared by sonication. This solution will be called "lysophosphatides", even though phosphatidylinositol and sphingomyelin are also present, and its concentration refers to the actual amount of total lysophospholipids it contained.

Dissection of axons. Two preparations of the squid (*Loligo pealii*) giant axon were used: one in which the giant axon was carefully freed from small nerve fibers and which will be referred to as "giant axon", and another containing the giant axon still surrounded by small adhering nerve fibers, which we shall refer to as "giant axon with fibers".[8, 16] The latter preparation weighs about eight times as much as the giant axon itself.

Treatment and recording of electrical activity of the squid giant axons. The axons having both ends ligated with threads were placed in chambers and covered with filtered natural sea water containing the agent used for treatment. The sea water was buffered with 1 mM Tris to pH 7·7 to 8·1. In certain experiments phospholipase D was dissolved in sea water at a pH of 5·6 (1 mM Na acetate buffer). After 30 min of incubation at a temperature of 16–20°, the solution in the chambers was removed and the axons were washed by circulating sea water.

Axonal conduction was measured with extracellular electrodes.[7, 8] Some of the axons were used for lipid extraction while others were exposed to ACh or curare as described before.[7, 8, 16]

Penetration of acetylcholine (N-*methyl*-^{14}*C*) *into the axoplasm.* After exposure of axons to experimental treatments or to normal sea water for 30 min, the axons were rinsed and placed for 1 hr in a solution containing ^{14}C-ACh plus nonradioactive

carrier to give a final concentration of $4{\cdot}5 \times 10^{-3}$ M. The solution also contained $2{\cdot}4 \times 10^{-4}$ M nonradioactive physostigmine salicylate in order to decrease enzymatic hydrolysis. The radioactivity of the final solution was about 8×10^5 dpm/ml. The axoplasm of the giant axon was then extruded and the per cent penetration of ACh into the axoplasm was calculated by comparing the disintegration values expected at equilibrium with the actual radioactivity found in the axoplasm.[9, 12, 13] Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter.

Extraction and chromatography of lipids. In a number of experiments we wanted to compare the action of the enzymes on axons with ligated ends and on axons with the ends left open. All axons were incubated with 2 ml of the enzyme solution in buffered sea water. The axons with ligated ends were then blotted on filter paper and rapidly weighed after cutting off the ligatures. Lipids were extracted by homogenization in chloroform:methanol (2:1, v/v) and washed with 0·04% $CaCl_2$ as described by Folch *et al.*[17] All the details have been given previously.[16] Since in the axons with open ends leakage of axoplasm was likely to occur, these axons were not removed from the enzyme solution; the entire incubate was extracted by homogenization with methanol and chloroform (3:1) as described by Marinetti *et al.* for extraction of plasma phospholipids.[19] The extract was then evaporated to dryness, reextracted in chloroform: methanol (2:1, v/v) and washed with 0·04% $CaCl_2$ solution. Aliquots of all lipid extracts were subjected to two-dimensional TLC and the spots were visualized in iodine vapors.[15, 16] Phosphorus content in the total lipid extracts and in the spots separated by chromatography was assayed by the method of Bartlett.[18]

Calculation of phospholipid splitting. The degree of phospholipid hydrolysis by phospholipase A was previously calculated by comparing the per cent distribution of every individual phospholipid in the treated axons with that in the controls.[15, 16] This was possible since the lysophospholipids resulting from phospholipase A action are quantitatively extracted by the method of Folch *et al.*[17] and satisfactorily recovered after TLC. The action of phospholipase C, in contrast, gives rise to phosphoryl choline, ethanolamine or serine, which are not extracted in chloroform: methanol, thus decreasing the ratio of lipid phosphorus per tissue weight whenever phospholipid splitting occurs. Since lipid phosphorus is lost, a calculation of per cent phospholipid distribution in the treated axons becomes meaningless. We therefore calculated for each weight of treated axons the "expected" amount of every individual phospholipid based on the phospholipid content and distribution in control axons,[16] which may be summarized as follows. The preparation of giant axon with fibers has a total lipid phosphorus (TLP) value of 0·38 μg/mg wet wt. and the following per cent distribution of phospholipids: lecithin (L), 52; phosphatidyl ethanolamine (PE), 27; phosphatidyl serine (PS), 7; phosphatidyl inositol (PI), 2; and sphingomyelin (SM), 12. The values for the giant axon alone were similar, except that the TLP was 0·23 and the values for L were about 5 per cent lower and those for PE about 5 per cent higher. The TLP in the axoplasm of the giant axon is much lower (0·09); however, the distribution of phospholipids is similar to that in the giant axon except that SM is absent and L values are about 10 per cent higher. These "expected" values were then corrected for the actual recovery from the silica plates (usually 80–90 per cent), compared with the experimental values and the differences expressed as per cent splitting. A sample calculation of the per cent splitting of lecithin by phospholipase C (2 mg/ml) in the giant axon with fibers might clarify the method used. A 1-ml lipid

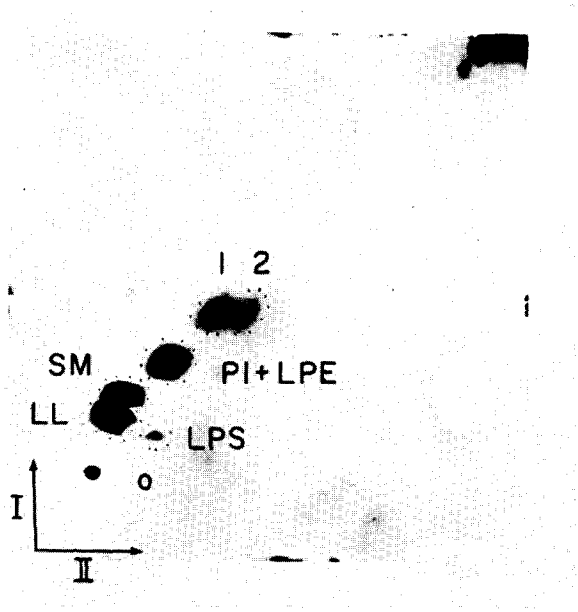

FIG. 1. Chromatographic pattern of a "lysophosphatide" extract. Giant axons with fibers were incubated overnight with 5 mg Ringhals venom/ml distilled water. Solvent system I: chloroform: methanol:water (65:25:4 by volume); solvent system II: 3-heptanone: acetic acid:water (80:50:10 by volume). Spots revealed by iodine vapor. Abbreviations: LL = lysolecithin; LPS = lysophosphatidyl serine; LPE = lysophosphatidyl ethanolamine; SM = sphingomyelin; PL = phosphatidyl inositol; O = origin. Spots 1 and 2, which are often seen in extracts from control axons, have no phosphorus. Since PI is not hydrolyzed by phospholipase A, it is possible to calculate the amount of LPE even though the PI and LPE spots overlap.[15]

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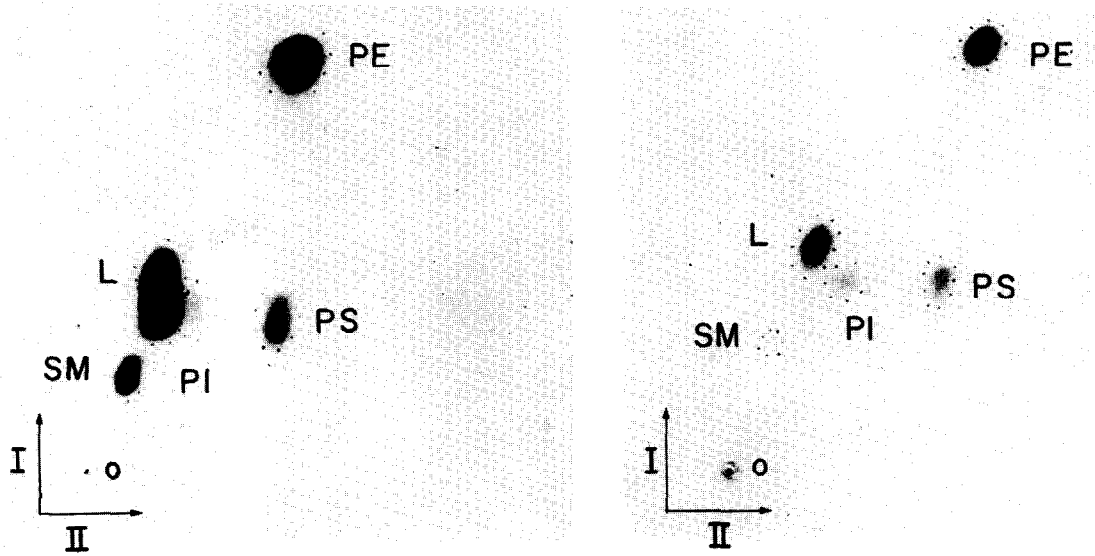

FIG. 2. Chromatographic pattern of phospholipids in control and in phospholipase C-treated axons. An equal aliquot of total lipid extract from equal weights of control (left) and phospholipase C-treated (right) axons was applied to the plates. Intact preparations of giant axons containing adhering small nerve fibers with their ends tied off were exposed to phospholipase C (20 mg/ml) for 30 min. Solvent systems and visualization are as in Fig. 1. Abbreviations: SM = sphingomyelin; L = lecithin; PI = phosphatidyl inositol; PS = phosphatidyl serine; PE = phosphatidyl ethanolamine; O = origin.

extract was obtained from 223 mg tissue. The total phosphorus recovery from a thin-layer plate spotted with 0·4 ml of the lipid extract was 16·6 μg, while the lecithin phosphorus value was 6·6μg. The total lipid phosphorus value directly determined on a separate aliquot of the extract was 0·215 μg/mg or 19·2 μg/0·4 ml spotted on the plate [0·215 $\times$ (223 $\times$ 0·4)] giving a per cent recovery of 87 [(16·6/19·2) $\times$ 100]. The expected amount of lecithin in this weight of tissue based on above control values is 17·6 μg [0·38 $\times$ (223 $\times$ 0·4) $\times$ 0·52]. The actual amount of lecithin on the plate was 7·6 μg (6·6/0·87) indicating a 57 per cent (17·6–7·6/17·6 $\times$ 100) splitting of lecithin by phospholipase C. The same type of calculation was used for the samples treated with phospholipase B, since glycerol phosphocholine, serine or ethanoline resulting from its action would not be extracted by chloroform–methanol.[20] Although phosphatidic acid produced by the action of phospholipase D should be present in the total lipid extract, we were not able to detect it on the chromatographic plate, so that we adopted the same procedure of calculation in the phospholipase D experiments as described above.

RESULTS

Effects of phospholipases on conduction and permeability of the squid giant axon. In contrast to the marked potency of phospholipase A,[15, 16] phospholipases B, C and D had no significant effect in 30 min on the height of the conducted action potential (less than 10 per cent decrease). The phospholipase B was tested in a concentration of 16 mg/ml in 3 experiments, and the phospholipases C and D in concentrations of 20 mg/ml in 6 and 4 experiments respectively. While phospholipase C itself had no effect on conduction, removal of the enzyme and return of the axon to fresh sea water caused a rapid deterioration in the action potential for reasons as yet unclear. Phospholipase D was equally inactive when tested at pH 7·8 and 5·6, although it has maximum enzymatic activity at the lower pH.[21] Sea water alone at this pH has no effect in 30 min on the action potential. Since phospholipase A when applied to a preparation of giant axons with fibers greatly increases the penetration of ^{14}C-ACh into the axoplasm of the giant axon and renders it sensitive to curare and ACh,[16] it was of interest to compare its effects with those of phospholipases B, C and D. Here again, a marked difference between the phospholipase A on one hand, and the phospholipases B, C and D on the other hand, was apparent. The per cent penetration of ^{14}C-ACh in axons treated with 0·05 mg/ml phospholipase A solution was 87 $\pm$ 12.[16] Treatment by the phospholipases B, C and D gave per cent penetration values similar to those found in control axons. The actual data are as follows; phospholipase B, 2 mg/ml, 4 $\pm$ 1%; phospholipase C, 20 mg/ml, 3 $\pm$ 0·3% and phospholipase D, 2 mg/ml, 2 $\pm$ 1% (2 experiments each); the control value was 3 $\pm$ 0·3% (10 experiments). In the experiments with phospholipase C, the enzyme and radioactive ACh were applied together because of previously noted deterioration of the axon after removal of phospholipase C. In addition to being unable to increase the penetration of ACh into the axons, phospholipases B, C and D were equally unable to render the axons sensitive to ACh or curare. After pretreatment with 16 mg/ml of phospholipase B, 20 mg/ml of phospholipase C or 20 mg/ml phospholipase D, 1·4 $\times$ 10^{-3} M curare or 4·5 $\times$ 10^{-3} M ACh was applied for 30–60 min (12 experiments). The decrease in spike height thus produced was less than 10 per cent, entirely similar to that noticed in control axons that were not subjected to pretreatment.

Hydrolysis of axonal phospholipids by phospholipases. It can be noted in Table 1 that an amount of phospholipase A (0·05 mg/ml) which blocks conduction and markedly increases the penetration of ^{14}C-labeled ACh split 1/3 to 1/2 of L and PE.[16] The sum of L and PE accounts for about 80 per cent of the total axonal phospholipids.[16] Phospholipase A does not hydrolyze SM and phosphatidyl inositol (PI). The TLP values are the same as those in control axons, as would be expected, since the lysophosphatides produced as a result of the hydrolysis of the β fatty acid ester are extracted by the chloroform–methanol procedure used.

TABLE 1. EFFECTS OF PHOSPHOLIPASES ON PHOSPHOLIPIDS IN SQUID AXONS*

Phospholipase	Concn (mg/ml)	Preparation	TLP (μg/mg wet wt.)	% Splitting†			
				L	PE	PS	SM
Ends of axons tied							
A	0·05	GAF	0·38 ± 0·02‡	48	37	10	0
	0·05	GA	0·23 ± 0·04	26 ± 12	39 ± 9		0
	0·05	Axoplasm		36 ± 5	50 ± 3		
B	16·0	GAF	0·36	14	4	0	4
C	2·0	GAF	0·23 ± 0·02	55 ± 1	29 ± 2	13 ± 0	52 ± 4
	20·0	GAF	0·17	84	50	3	84
	20·0	GA	0·17	56	27	7	100
	20·0	Axoplasm	0·07	54	21		
	2·0§	GAF	0·14	95	86	30	69
D	20·0*	GAF	0·33	20	9	0	7
Ends of axons open							
B	2·7	GAF	0·28	9	9	15	15
C	2·0	GAF	0·16 ± 0·01	69 ± 4	48 ± 1	17 ± 9	74 ± 3
	2·0	GAF	0·10	64	57	33	100
D	2·0	GAF	0·31	10	17	3	7

* All incubations at pH 7·6 to 8·0 except for the experiment with phospholipase D, which was at pH 5·6.

† Values for % splitting were calculated as described in Methods. For comparison, results previously obtained with phospholipase A[16] are recorded below. GA = giant axon free of nerve fibers; GAF = giant axon surrounded by small nerve fibers; TLP = total lipid phosphorus; L = lecithin; PE = phosphatidyl ethanolamine; PS = phosphatidyl serine; SM = sphingomyelin.

‡ Results shown as mean ± S.E. are based on 2 or 3 experiments.

§ Axon was homogenized in the presence of phospholipase C.

Under the conditions of our experiments, phospholipases B and D were relatively inactive, never splitting more than 20 per cent of the individual phospholipids. Phospholipase D was tested both at the usual pH of sea water ($\sim$ 7·8) and at pH 5·6. PI was not hydrolyzed by either enzyme. The TLP values in the phospholipase D experiments are slightly lower than the control values even though we would have expected that phosphatidic acid, the product of hydrolysis by phospholipase D, would be extracted with the chloroform–methanol extraction procedure.

The results obtained with phospholipase C are of special interest because of the extensive phospholipid hydrolysis by concentrations of this enzyme (2 and 20 mg/ml) which neither block conduction nor increase permeability to ACh. The extent of phospholipid hydrolysis is indicated both in the actual per cent splitting values listed as well as in the TLP values. The TLP values are much lower than the control values, since the phosphorylated water-soluble compounds (phosphoryl choline, phosphoryl ethanolamine *etc.*), which are the products of hydrolysis by phospholipase C, are not

extracted by the lipid extraction procedure used. A comparison of the lipid extract from control and phospholipase C-treated axons is shown in Fig. 2. An equal aliquot of the total lipid extract from an equal weight of control and phospholipase C-treated axon was applied to each plate so that the decrease in size of the phospholipid spots represents the extent of splitting by the enzyme. The diglycerides and phosphorylated bases produced by the action of this enzyme are not visualized on the plates, the percentage splitting being calculated as described under Methods. The extent of phospholipid splitting by phospholipase C appeared similar in preparations of giant axons free of all adhering small nerve fibers and in preparations containing adhering small nerve fibers. PS was more resistant to the action of phospholipase C than the other three phospholipids listed in Table 1. PI was not hydrolyzed by phospholipase C. The phospholipid hydrolysis by 2 mg/ml of phospholipase C in axons where the ends were open was about equal to that of 20 mg/ml on axons where the ends were tied. Apparently there is some permeability barrier to the penetration of phospholipase C through the axonal membranes, as evidenced also by the very high phospholipid splitting induced by the enzyme in a homogenized preparation (Table 1).

Effects of products of phospholipase A action. Earlier studies had shown that the extent of phospholipid splitting by phospholipase A was about equal in preparations of giant axons with or without adhering small nerve fibers (ref. 16 and Table 1), although phospholipase A only blocked conduction and increased permeability in preparations containing the fibers.[16] We have also observed (Table 1) that extensive phospholipid splitting, as produced by phospholipase C, is compatible with maintenance of axonal conduction and membrane permeability. It therefore appeared of interest to determine whether the lytic products arising as a result of the action of phospholipase A on membranal phospholipids could be responsible for the effects on conduction and permeability caused by phospholipase A.

Emulsions of oleic and linoleic acid (1 %, v/v sea water) and a 1 % suspension of stearic acid had no effect in 30 min on conduction of the giant axon (6 experiments). As further indication that evolved fatty acids are not responsible for the action of phospholipase A, it was found that 4 mg/ml of human serum albumin, which is known to strongly bind free fatty acids, did not prevent irreversible block of conduction induced in 20 min by cottonmouth moccasin venom (0·1 mg/ml) (2 experiments). Human serum albumin (4 mg/ml) itself had no effect on conduction in 60 min.

In order to check whether the other products of phospholipase A action, i.e. lysophosphatides, could be responsible for the effects of phospholipase A, we applied a purified preparation of lysolecithin to axonal preparations (Table 2). In a concentration of 0·5 mg/ml, lysolecithin blocked conduction in preparations of giant axons with or without adhering small nerve fibers. About 10 min prior to block of conduction, lysolecithin (0·5 mg/ml) caused repetitive firings of the giant axon at a rate of about 200/sec. Lysolecithin (0·5 mg/ml) also markedly increased the permeability of the giant axon in both types of preparations to ^{14}C-ACh. As previously noted, the penetration of ACh in control axons is about 3 per cent. Even this low level of apparent penetration may be due to contamination during the process of extrusion or to the presence of small amounts of lipid-soluble impurities in the preparation of ^{14}C-ACh.[9] After the application of lysolecithin in a concentration of 0·1 or 0·2 mg/ml, to giant axons with fibers, the effects of $1{\cdot}4 \times 10^{-3}$ M curare and $4{\cdot}5 \times 10^{-3}$ M ACh were checked. These two compounds produced only about a 20 per cent decrease in

spike height in 30 min (7 experiments), which agrees with the inability of these concentrations of lysolecithin to increase permeability (Table 2). Previous studies have shown that the inability of ACh and curare to affect conduction in the giant axon may be related to their inability to penetrate through the membranes of this axonal preparation.[9]

TABLE 2. EFFECTS OF LYSOLECITHIN AND A LYSOPHOSPHATIDE MIXTURE ON CONDUCTION AND PERMEABILITY OF THE SQUID GIANT AXON*

Treatment	Concn (mg/ml)	Time (min)	Preparation	% Decrease AP	% Penetration of ACh
Lysolecithin	0·1	30	GAF	8 ± 10 (3)	4 ± 2 (2)
	0·2	30	GAF	17 ± 4 (4)	4 ± 1 (2)
	0·5	15–30	GAF	100 ± 0 (7)	33 ± 1 (2)
	1·0	10	GAF	100 ± 0 (2)	57 ± 13 (2)
	0·1	30	GA	10 ± 8 (4)	3 ± 1 (2)
	0·5	10	GA	100 ± 0 (5)	24 ± 3 (2)
Lysophosphatides	0·2	30	GAF	7 ± 3 (9)	4 ± 1 (2)
	0·5	20–30	GAF	100 ± 0 (5)	23 ± 3 (2)
	0·1	30	GA	15 ± 5 (2)	
	0·5	5–10	GA	100 ± 0 (2)	

* Results are presented as means ± S.E. Number of experiments is shown in parentheses. After treatment of axons with lysophospholipids, *N*-methyl^{14}C-ACh was applied for 1 hr, after which the axoplasm of the giant axon was extruded (see Methods). AP = action potential; GA = giant axon free of nerve fibers; GAF = giant axon surrounded by small nerve fibers; ACh = acetylcholine.

As a further test of whether evolved lyso products are a major factor in the course of events following venom or phospholipase A treatment, we tested the effects of a mixture of "lysophosphatides" (see Methods). The concentration of lysophosphatides required to block conduction and increase penetration of ^{14}C-ACh was identical to that of purified lysolecithin, i.e. 0·5 mg/ml. Like the lysolecithin, the mixture of lysophosphatides also blocked conduction in giant axons free of adhering nerve fibers. After exposure of axons for 30 min to lysophosphatides (0·2 mg/ml), $1{\cdot}4 \times 10^{-3}$ M curare caused a 43 ± 14 per cent decrease in spike height in 30 min (7 experiments). This effect of curare is less than that produced after venom or phospholipase A treatment of the axon, but is considerably greater than the complete inactivity of curare in control axons.[7, 8, 16]

In order to check whether some contamination with active venom might have occurred during the extraction of the lysophosphatides (see Methods), a similar extraction was applied to a mixture of venom and lipid-free egg albumin. This control was completely inert on the giant axon. Similarly, a control of homogenized axons, incubated overnight in the absence of venom and extracted as before, was completely inert, thus showing that none of the native axonal phospholipids has any effect on conduction.

DISCUSSION

In analyzing the function of any particular membrane component, it may be useful to apply to the membrane agents which will selectively bind, extract or catabolize it and then determine if the membrane is still able to carry on its normal functions. By carrying out such a "chemical dissection" of the mitochondrial membrane, Green

and Fleischer were able to conclude that phospholipid is an integral part of the membrane, providing a medium of low dielectric constant in which reactions abetted by such a medium can proceed.[22]

Treatment by phospholipases has become a method of choice in determining the function of the phospholipids in membranes. In his studies on the lobster nerve, Tobias used heated snake venom as the source of phospholipase A and concluded that nerve membrane resistance and capacitance are more likely to be associated with phospholipid than with protein.[6] The phospholipase A was also found able to increase permeability and block conduction in squid and lobster axons[15, 16] as well as in eel electroplax.[23] One might therefore conclude that phospholipids are essential for the normal functioning of these preparations. However, in all the studies using phospholipase A, one should consider the fact that the products of phospholopid hydrolysis, i.e. lysophosphatides and fatty acids, are themselves lytic, hemolyzing red blood cells, blocking conduction in lobster nerve, causing demyelination in the central nervous system and increasing permeability of whole cell preparations.[24–27] One should therefore try to dissociate the effects of the phospholipid splitting *per se* and the secondary effects of the products evolved. Indeed, Tobias suggests that block of conduction in lobster nerve by heated venom might be due to the formation of lysophosphatides.[25] Other investigators, however, suggested that uncoupling of oxidative phosphorylation,[28] morphological alterations of mitochondria[29] and increased permeability of brain and muscle[30] are due to direct action of phospholipase A and not due to the liberation of lytic products. These conclusions were reached on the basis of the expected small quantities of lysophosphatides to be produced as a result of phospholipase A action,[28] differing effect of phospholipase A and lysophosphatides[29] or similar effects being produced by phospholipase C whose action does not give rise to lysophosphatides or fatty acids.[30]

In a previous study on squid giant axons, we made the suggestion that the products of phospholipid hydrolysis by phospholipase A might be of major importance in the overall effects observed. That suggestion was based on the observation that the phospholipase A affected conduction and permeability of the squid giant axon with fibers, whereas the enzyme was inert on giant axons free of fibers even though the extent of phospholipid splitting was similar in both types of preparations.[16] In addition, electron microscopic studies showed that both phospholipase A and a cationic detergent produced marked vesicularization and disruption of the Schwann cell when they were applied to giant axons with fibers, whereas phospholipase A caused no morphological alterations when applied to giant axons free of surrounding fibers.[31]

In an attempt to further clarify the role of the lyso products, we now investigated the effects of phospholipase C which, unlike the phospholipase A, gives rise to diglycerides and phosphorylated nitrogen-containing bases, neither of which have detergent properties. As reported in this study, phospholipase C affects neither conduction nor permeability of the squid giant axon even though the extent of phospholipid splitting is much greater than that observed with concentrations of phospholipase A which block conduction and increase permeability. In contrast, lysolecithin and a mixture of lysophosphatides blocked conduction and increased permeability in giant axons whether or not they were surrounded by adhering small nerve fibers, similarly to findings[7, 9] with another detergent. Lysophosphatides in our studies, however, were not as effective as venom or phospholipase A in rendering

axons sensitive to curare or acetylcholine. The penetration studies with ^{14}C-acetylcholine, however, indicated that, in contrast to findings with phospholipase A,[16] it was necessary to use blocking concentrations of lysophosphatides in order to increase permeability. One can apparently therefore obtain more effective disruption of barriers if the lysophosphatides are produced *in situ* through an enzymatic reaction than if they are added externally. Free fatty acids, the other product of phospholipase A action, were, in contrast, inert. These findings together with our earlier studies[8, 16] demonstrate that the actions of phospholipase A on the squid giant axon are due to evolved lysophosphatides and not to phospholipid splitting *per se*. In marked contrast, conduction of lobster giant axons is blocked by phospholipases A and C,[25] as are the walking leg nerves of lobster.[15, 32] At this time no explanation can be offered as to why the action of phospholipase C differs in lobster and squid axons.

The results with phospholipase C are of special interest in regard to the essentiality of phospholipids for the maintenance of structural and bioelectrogenic properties of membranes. Since phospholipids have both hydrophilic and lipophilic moieties, they would appear admirably suited to bridge or bind hydrophilic proteins in the membrane with lipids such as cholesterol. In addition, there has been considerable recent speculation that phospholipids are likely candidates for the function of receptors. Goldman has proposed for example that phosphate groups in the membrane act as exchange sites through which ions pass and may be intimately involved in the genesis of bioelectricity.[2] It appears to us therefore of special interest that axonal conduction and membrane permeability can be maintained in the presence of extensive phospholipid splitting by phospholipase C, reaching up to 100 per cent splitting of sphingomyelin, 84 per cent splitting of lecithin and 50 per cent splitting of phosphatidyl ethanolamine, which are the three major phospholipids in the preparation of squid axons. We can tentatively conclude therefore that sphingomyelin is not essential for the maintenance of the structural and functional permeability properties of the squid axon. Concerning the other individual phospholipids, any conclusion would remain speculative since we cannot yet distinguish between the extent of hydrolysis in the excitable membrane proper and that occurring in the Schwann cell or in the connective tissue. However, analysis of axoplasm extruded from phospholipase C-treated axons shows considerable splitting of lecithin and phosphatidyl ethanolamine, thus indicating that hydrolysis of phospholipids in the axolemma might have occurred.

Electron microscopic studies are currently in progress to determine whether phospholipase C or lysophosphatides have any effects on the structure of the giant axon. Earlier investigations have shown that both phospholipase A and a cationic detergent cause marked disruption of the Schwann cell surrounding the squid giant axon.[31]

Our results with phospholipase C may also indicate something about the structural organization of membranes. The Davson–Danielli–Robertson type model of the membranes has a phospholipid bilayer with the polar heads oriented outward and surrounded by protein on both sides.[33] In this model the major forces of binding between phospholipid and protein are electrostatic. Another possible structure which has been suggested[34, 35] is with the polar heads of the phospholipids and the charged groups of the proteins at the surfaces of the membrane. The interior of the membrane contains the rest of the protein and the hydrophobic tails of the phospholipids. In this model hydrophobic interactions rather than electrostatic forces would be responsible

for holding the membrane constituents together. Our results with phospholipase C appear to be in support of this latter model, since the phosphoester bands in the intact membrane are readily susceptible to the enzyme molecule and hydrolysis of the polar heads does not alter conduction or permeability of the membrane, which would be expected to be altered if electrostatic binding was of major importance. Lenard and Singer[36] have similarly observed that release of about 70 per cent of the total red cell membrane phosphorus with phospholipase C does not alter membrane integrity or average protein conformation. They also take this as evidence that the charged groups of the phospholipids are at the surface of the membrane. On the other hand, our results with phospholipase A on the giant axon free of small nerve fibers are difficult to interpret by either model. According to one model, the enzyme would have had to penetrate through a protein layer before being able to hydrolyze the β fatty acid ester, while according to the other model, we would have expected hydrolysis to be accompanied by a disruption of hydrophobic binding forces between lipid and protein, which might have been expected to alter conduction or membrane permeability. It is possible, however, that the hydrophobic interaction of protein and the fatty acid at the α position of the phospholipid is sufficient to maintain membrane integrity.

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