BBA 4094

FACTORS IN VENOMS LEADING TO BLOCK OF AXONAL CONDUCTION BY CURARE

PHILIP ROSENBERG AND K. Y. NG

Departments of Neurology and Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

(Received February 18th, 1963)

SUMMARY

Several enzyme and other fractions of venoms were studied to determine the component (or components) responsible for rendering axonal conduction sensitive to the action of acetylcholine and curare. A phospholipase A (EC 3.1.1.4)-rich fraction from cobra venom and phospholipase D (EC 3.1.4.4) rendered the squid axon sensitive to the action of curare. Venom phosphodiesterase (EC 3.1.4.1), apparently also active, was found to have considerable phospholipase A activity. A phospholipase A-poor fraction of cobra venom, L-amino acid oxidase (EC 1.4.3.2), Cobroxin, lysolecithin and phospholipase C (EC 3.1.4.3) were all ineffective in rendering curare active on the squid axon. All the enzymes and venom products tested were relatively weak in their direct effects on conduction except for the phospholipase A-rich fraction. Heating a solution of cottonmouth venom at an alkaline pH destroyed almost all of its phospholipase A activity whereas heating at an acid pH had little effect on this enzymic activity. Eastern diamondback rattlesnake venom hydrolyzes egg lecithin at a rate only slightly less than that of cottonmouth venom. The ability of several venoms to hydrolyze beef lecithin was also compared. The results support the assumption that the phospholipase A of venoms is responsible for their ability to increase the permeability of the squid axon allowing thereby acetylcholine and curare to penetrate into the axon and to affect conduction.

INTRODUCTION

Pretreatment of the squid giant axon with certain snake venoms, in concentrations which have no effects of their own, renders ACh and (+)-tubocurarine (curare) effective in blocking conduction[1-3]. Evidence has been offered that the venoms act by increasing the permeability of this preparation to normally impenetrable compounds such as ACh and curare[4]. The results offer new support to the assumption that these compounds affect conduction by interaction with the ACh system in the neuronal membrane as suggested by NACHMANSOHN's theory[5,6].

In addition to non-enzymic components, venoms contain many enzymes including phospholipase A (EC 3.1.1.4), hyaluronidase (EC 4.2.99.1), proteolytic enzymes, L-amino acid oxidase (EC 1.4.3.2), phosphodiesterase (EC 3.1.4.1) etc.[7-12]. Venoms of the elapidae, such as cobra venom contain cholinesterase (EC 3.1.1.8)

Abbreviation: ACh, acetylcholine.

activity[13,14]. It is obviously of great interest to determine the component (or components) of the venoms responsible for increasing the permeability of the squid giant axon preparation. Earlier studies, such as the use of certain enzymes[1], heating of venoms at alkaline and acid pH[2], and the effects of venoms on finely and crudely dissected axons[2], indicate that phospholipase A of the venoms might be reacting with a phospholipid of the membrane, probably not lecithin, to yield a lysophosphatide which is then responsible for disrupting permeability barriers. However, the evidence was still incomplete and various additional experiments appeared desirable for further elucidating the mechanism of action of the venoms. The results obtained further strengthen the supposition that phospholipase A is the active component of the venoms responsible for the effects on axons described.

METHODS AND MATERIALS

Measurements of electrical activity with extracellular electrodes were performed under conditions identical to those previously described[2,3]. The giant axons of the squid (*Loligo pealii*) were dissected with removal of many, but not all of the fine fibers of the stellar nerve[2,3]. The procedure used for determining whether the enzymes or venom fractions rendered curare active is the same as that previously used[1-3]. All decreases of action potential are recorded in the tables as means ± S.E. (standard error or standard deviation of the mean)[1,3]. Reversibility estimated as previously described[2], was checked over a period of 20–40 min unless otherwise stated.

Phospholipase A activity was measured by the procedure of MAGEE AND THOMPSON[15], using an aqueous 2,4,6-collidine buffer system containing ether. 1 mg of phospholipid was routinely used as substrate in a total volume of 1 ml incubation mixture (pH 7.8; 18–22°). Controls were run with either no phospholipid or no enzyme. Ether (0.5 ml) was added to all spectrophotometer tubes immediately before checking absorbancy to remove the cloudiness which sometimes developed in the tubes. To determine the effects of heating upon phospholipase A activity, cottonmouth venom was heated at pH 5.5 or 8.5 as previously described[2]. All phospholipase A activities are presented as μg phospholipid hydrolyzed (mean ± S.E.). Complete hydrolysis would be 1 mg. Egg lecithin contains 2 moles of ester per gramatom of phosphorus, only one of which is hydrolyzable by phospholipase A. Beef lecithin is a mixture of lecithin (phosphatidylcholine) and choline plasmalogen (phosphatidalcholine). The ratio of α,β-unsaturated ether to phosphorus indicated that our sample of beef lecithin contained about 60% plasmalogen which agrees with earlier results by GOTTFRIED AND RAPPORT[16]. Unsaturated-ether content was determined by method described by above investigators[16], while phosphorus was checked by method of BEVERIDGE AND JOHNSON[17]. The method used for determining phospholipase A activity measures the decrease in ester bonds so that complete hydrolysis of egg lecithin causes a 50% decrease in absorbancy whereas complete hydrolysis of beef lecithin gives about a 70% decrease in absorbancy.

The following lyophilized snake venoms with their identifying lot numbers listed below were purchased from Ross Allen Reptile Institute, Silver Springs, Fla. *Agkistrodon p. piscivorus* (cottonmouth moccasin) 6-29-61; *Crotalus adamanteus* (Eastern diamondback rattlesnake) 2-2-61; *Naja naja* (hooded cobra) 959 L (1961); *Vipera russelli* (Russells viper) 3-3-61; *Ophiophagus hannah* (king cobra) 61L (1961). Phospha-

tidylethanolamine, phosphatidylserine and DL-α-cephalin (synthetic) were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. L-α-Lecithin, β,γ-dipalmitoyl (synthetic) was purchased from Mann Research Lab., New York, N.Y. Solutions of egg and beef lecithin in absolute ethanol were purchased from Sylvana Chemical Co., Orange, N.J. The egg lecithin (Control No. EL-30X) had 36.6 mg lecithin per ml of ethanol while beef lecithin (L-80) had 38.3 mg of phospholipids per ml. L-α-Cephalin (Chrom. pure-2355B), lysolecithin (2551 D) and phospholipase D (EC 3.1.4.4) (2552D) were purchased from General Biochemicals, Chagrin Falls, Ohio, which prepared the lysolecithin from egg lecithin by hydrolysis with snake venom, and purified it by column chromatography. It is primarily the α-isomer and contains mainly palmitic and stearic acids in ratio of 6:4, and has an ester:P ratio of 1.0. Phospholipase D, a lyophilized preparation isolated from cabbage was stated to be almost salt-free and standardized to have 0.5 units/mg activity. L-Amino acid oxidase (Venom lot No. 27), venom phosphodiesterase (lot No. 23) and phospholipase C (EC 3.1.4.3) (No. 6204) were purchased from Worthington Biochemical Corp., Freehold, N.J. The stable powdered oxidase was stated as being prepared by the procedure of WELLNER AND MEISTER[18], and deaminating 4.56 μmoles L-leucine per min per mg under specified conditions. Lyophilized phosphodiesterase prepared from *Crotalus adamanteus* venom[19] was stated to have an activity of 0.465 per min under their specified conditions of assay. A sample of phosphodiesterase (lot 107-7/2/62) was kindly provided by Dr. F. E. RUSSELL, Laboratory of Neurological Research, Loma Linda School of Medicine, Los Angeles, Calif. The results with this sample of phosphodiesterase and that from Worthington Biochemical Corp. were similar and were combined to give those shown in the tables and figures. Phospholipase C is a partially purified α-toxin from *Clostridium welchii* with a stated activity of 20 mg phosphorus liberated per mg per 30 min from egg lecithin at 37.5°. Cobroxin powder was kindly supplied by Hynson, Westcott and Dunning, Inc., Baltimore, Md. According to the company, Cobroxin is prepared from crude cobra venom by a series of fractional precipitations, whereby the hemolytic and proteolytic factors are almost entirely removed. 1 mg of this product can kill about 200 mice in 24 h (200 mouse units), whereas crude venom has about 100 mouse units/mg. An inhibitor of phospholipase A and D (see refs. 20, 21), 2,3-distearoyloxypropyldimethyl-β-hydroxyethylammonium acetate, was kindly supplied to us by Dr. R. P. GEYER, Harvard University School of Public Health, Boston, N.Y. Stable aqueous dispersions were prepared in the manner described by ROSENTHAL AND GEYER[20]. Dialyzable (poor in phospholipase A) and non-dialyzable (rich in phospholipase A) fractions of cobra venom were generously supplied to us by Drs. LAL and SUMYK of the Armour Research Foundation, Illinois Institute of Technology, Chicago, Ill. Dr. LAL found that the dialyzable fraction caused little hemolysis even in a concentration 300-fold as great as the concentration of the non-dialyzable fraction causing about 75 % hemolysis. Crystalline (+)-tubocurarine chloride was purchased from K and K Laboratories, New York, N.Y.

RESULTS

Recordings of action potential

The direct effects upon electrical activity of various venom fractions, enzymes and phospholipids are shown in Table I. Reversibility was checked with the phospho-

lipase A-rich fraction for periods up to 2 h. Lysolecithin in 100 μg/ml is inactive (2 experiments) but in 500 μg/ml it had an effect. In addition to egg and beef lecithin we also tested the effects on electrical activity of phosphatidylethanolamine, phosphatidylserine, L-α-cephalin and L-α-lecithin. Saturated solutions of each of the above compounds had no effect on electrical activity in 30 min (2 experiments with each compound). 0.5 mg/ml of 2,3-distearoyloxypropyldimethyl-β-hydroxyethylammonium acetate which is an inhibitor of phospholipase A and D (see MATERIALS) had no effect on spike height in 30 min (2 experiments). This inhibitor in a concentration of 500 μg/ml did not markedly antagonize the action of 50 μg/ml of cottonmouth venom. In 4 experiments the combination caused a 78 ± 14 % irreversible decrease in the action potential in 34 ± 9 min, while 50 μg/ml of cottonmouth venom alone caused a 90–100 % decrease in the action potential in about 30 min[2,3]. However, under our conditions of measurement the inhibitor did not antagonize the hydrolysis of egg or beef lecithin by cottonmouth venom (see *Phospholipase A measurements*).

Table II lists the compounds tested as to their ability to render axons sensitive to 1.4 mM curare while controls are not affected by 5.6 mM[1-3]. The pretreatment agents were applied for 30 min except for 4 μg/ml of the phospholipase A-rich fraction which was applied for 20 min, and 100 μg/ml of the phospholipase A-poor fraction which was applied for about 50 min. During the time of pretreatment the following

TABLE I

THE DIRECT EFFECTS OF SEVERAL ENZYMES, VENOM FRACTIONS AND PHOSPHOLIPIDS ON THE ACTION POTENTIAL OF THE SQUID GIANT AXON

Results are given as means ± S.E. of the mean.

Compound	*Concn. (μg/ml)*	*No. of expts.*	*Exposure (min)*	*Action potential (% decrease)*	*% reversibility*
Phospholipase A-rich fraction	2	6	30 ± 0	5 ± 2	0
	10	4	30 ± 0	50 ± 25	0
	30	4	23 ± 1	100 ± 0	0
	100	2	10 ± 0	100 ± 0	0
Phospholipase A-poor fraction	100	6	42 ± 7	26 ± 7	—
	200	2	50 ± 0	100 ± 0	0
Phospholipase C	200	2	25 ± 5	2 ± 2	—
Phospholipase D	10	4	30 ± 0	0 ± 0	—
	100	3	28 ± 10	8 ± 11	—
Phosphodiesterase	100	4	58 ± 3	94 ± 6	0
L-Amino acid oxidase	100	4	30 ± 0	20 ± 11	—
Cobroxin	100	2	60 ± 0	7 ± 7	—
	500	4	50 ± 6	90 ± 11	0
Lysolecithin	100	2	25 ± 5	0 ± 0	—
	500	4	20 ± 4	79 ± 12	0
Egg lecithin	500	2	20 ± 0	0 ± 0	—
	1000*	2	25 ± 5	0 ± 0	—
Beef lecithin	1000*	2	30 ± 0	8 ± 0	—

* A solution of 1 mg lecithin per ml of sea water also contains about 2.6 % ethanol.

TABLE II

EFFECT OF CURARE ON THE ACTION POTENTIAL OF THE GIANT AXON OF SQUID FOLLOWING PRETREATMENT WITH VARIOUS ENZYMES AND VENOM FRACTIONS

Results are given as means ± S.E. of the mean.

Pretreatment	Concn. (μg/ml)	No. of expts.	Curare (1.4 mM)		% reversibility
			Exposure (min)	Action potential (% decrease)	
Phospholipase A-rich fraction	2	3	23 ± 4	83 ± 17	37 ± 19
	3	3	15 ± 8	73 ± 8	—
	4	2	5 ± 3	96 ± 4	91 ± 9
Phospholipase A-poor fraction	100	6	30 ± 0	23 ± 8	—
Phospholipase C	500	4	30 ± 0	12 ± 5	—
Phospholipase D	10	2	30 ± 0	58 ± 8	0
	100	4	20 ± 4	73 ± 16	0
	500	3	18 ± 7	86 ± 14	0
Phosphodiesterase	20	4	25 ± 5	42 ± 20	—
	100	6	13 ± 6	70 ± 15	0
L-Amino acid oxidase	100	4	30 ± 0	3 ± 16	—
Cobroxin	200	4	30 ± 0	5 ± 3	—
Lysolecithin	100	3	30 ± 0	14 ± 17	—

agents caused less than a 10 % decrease in spike height: phospholipase C, Cobroxin, lysolecithin, and the lowest concentrations listed of phosphodiesterase, phospholipase D and the phospholipase A-rich fraction of cobra venom. The other pretreatments listed caused a 15–25 % decrease in spike height. Following the pretreatment procedure the axons were rinsed for 10 min in normal sea water, then curare was applied. Attempts to observe reversibility of the curare effect after phospholipase D were made for 60 min.

We also tested the effect of 1.4 mM curare following a 30-min pretreatment of the squid axon with 500 μg/ml of the phospholipase A and D inhibitor (see MATERIALS) plus 15 μg/ml of cottonmouth venom (3 experiments). The combination of these pretreatment agents had no effect on spike height, but curare applied following them caused an 83 ± 17 % decrease in the action potential in 15 ± 8 min. This effect was reversible to the extent of 59 ± 15 %. The inhibitor, therefore, did not prevent cottonmouth venom from rendering curare active; this is consistent with the observation that under the conditions used this agent did not inhibit the phospholipase A activity of cottonmouth venom (see *Phospholipase A measurements*).

Phospholipase D, phosphodiesterase and the phospholipase A-rich fraction from venom appeared to render curare active, although reversibility was only obtained with the phospholipase-rich fraction. One of the two experiments with 4 μg/ml of the phospholipase A-rich fraction is shown in Fig. 1. Block of conduction by curare was rapid and reversible. The results with the phospholipase-rich fraction appeared so promising that a series of 8 experiments were performed to see if ACh was rendered

active by this pretreatment. However, following treatment with this fraction of cobra venom 4.4 mM ACh caused only about a 50% decrease in spike height in 100 min and 0.44 mM ACh had no effect on spike height. These effects with ACh are not as strong as had been obtained following cottonmouth venom[2,3].

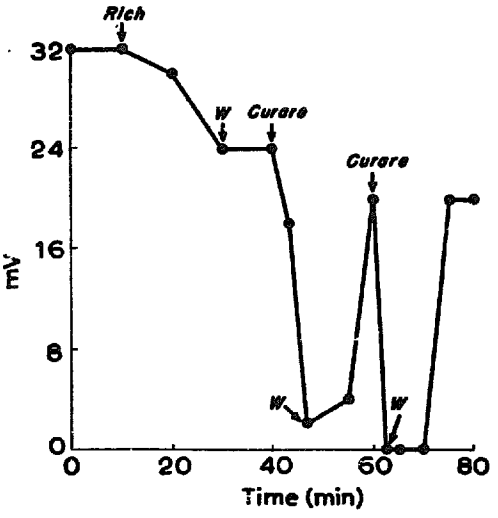


Fig. 1. Effect of 1.4 mM curare on the action potential of the squid giant axon following pretreatment with 4 μg/ml of phospholipase A-rich fraction (Rich) from cobra venom. W indicates return to normal sea water.

It was suggested that cottonmouth venom may have rendered curare and ACh active by liberating a lysophosphatide in the membrane which was then responsible for disruption of permeability barriers[2]. To test this idea, groups of 8 axons were homogenized or placed intact in 5 ml of sea water which in some cases contained 25 μg/ml cottonmouth venom. After 6 h the 5 ml of sea water was diluted to 25 ml and tested for effects on electrical activity of the squid axon. At the end of this 6-h incubation the intact axons were also homogenized and tested for effects on electrical activity. Neither the incubates nor the homogenized axons had any effect on electrical activity in 30 min (12 experiments). In another series of experiments egg or beef lecithin 1 mg/ml was incubated for 4 h with 25 μg/ml of cottonmouth venom dissolved in either sea water or a collidine buffer–ether mixture[15]. No potent product of venom action was found under the conditions used (8 experiments).

Phospholipase A measurements

Using the reaction mixture of MAGEE AND THOMPSON[15] their observation was confirmed that lysolecithin is not hydrolyzed by cottonmouth venom. In most of the measurements of phospholipase A activity egg and beef lecithin were used rather than purified substrates because the hydrolysis was slower and incomplete with purified substrates. According to CONDREA *et al.*[22] snake-venom phospholipase A splits egg yolk and serum phospholipids at a rate 10–20-fold greater than obtained with purified ovolecithin preparations. Several experiments were performed using phosphatidylserine, phosphatidylethanolamine and DL-α-cephalin as substrate and cottonmouth or Eastern diamondback rattlesnake venom (50 μg/ml) as enzyme sources. In 40 min only about 30% of these substrates were hydrolysed which is less than obtained with egg or beef lecithin. There was no difference in the hydrolysis

of these substrates by cottonmouth or rattlesnake venom except with phosphatidylethanolamine as substrate with which rattlesnake venom appeared about one-third as potent as cottonmouth.

TABLE III

HYDROLYSIS OF EGG LECITHIN (1 mg) BY 50 μg OF VARIOUS ENZYMES AND 50 μg OF COBROXIN

Results are given as means ± S.E. of the mean.

Preparation	*μg lecithin hydrolyzed per*		
	10 min	*20 min*	*40 min*
Phosphodiesterase	279 ± 23	433 ± 25	726 ± 21
Phospholipase C	177 ± 9	—	196 ± 21
Phospholipase D	14 ± 14	28 ± 14	0 ± 0
L-Amino acid oxidase	82 ± 23	95 ± 27	95 ± 13
Cobroxin	111 ± 14	—	153 ± 14

Each measurement of μg lecithin hydrolyzed (Table III, Fig. 2–5) is based on 3 or 6 experiments. Complete hydrolysis even with egg and beef lecithin, was usually not observed within the time period of incubation possibly because we worked at room temperature and pH 7.7. MAGEE AND THOMPSON[15] observed complete hydrolysis by venom at 30° and pH 6–6.5; however, at pH 8 using the same concentration of venom the reaction only went to about 85 % of completion.

Heating at an alkaline pH destroyed the direct potency of cottonmouth and hooded cobra venom and also destroyed their ability to render curare active[2]. In contrast, heating at an acid pH did not destroy the venom effects. It has been reported

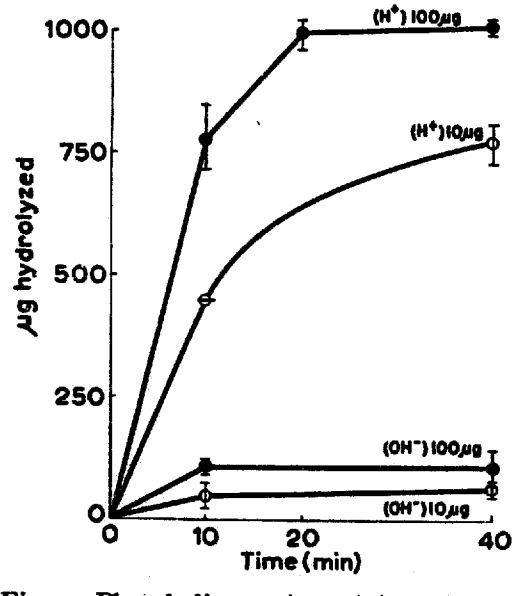


Fig. 2. Phospholipase A activity of cottonmouth-moccasin venom heated at pH 5.5 (H^+) and pH 8.5 (OH^-). Results are shown as means ± S.E. of the mean. Egg lecithin (1 mg) was the substrate.

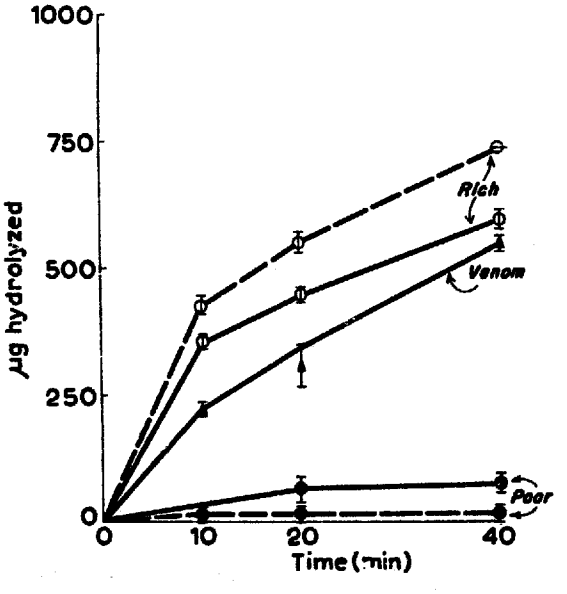


Fig. 3. Phospholipase A activities of 5 μg of phospholipase A-rich (Rich) and 100 μg of phospholipase-poor (Poor) fractions of cobra venom and activity of 5 μg of hooded cobra venom (Venom). 1 mg of egg (—) or beef (– – –) lecithin was the substrate. Results are shown as means ± S.E. of the mean.

by several authors[15,23,24] that alkaline heating destroys phospholipase A activity while the enzyme is resistant to boiling at an acid pH. This effect has been checked with the sample of cottonmouth venom used and with conditions of heating identical to that used when effects on electrical activity were tested[2]. The phospholipase A activity of cottonmouth venom upon heating is shown in Fig. 2.

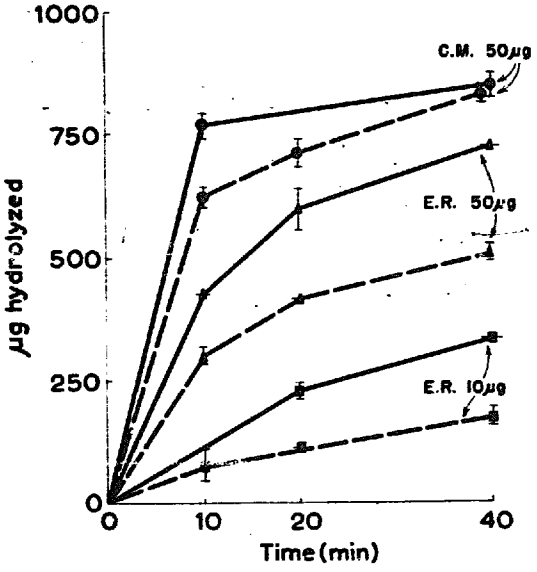


Fig. 4. Phospholipase A activity of cottonmouth-moccasin (C.M.) and Eastern diamondback rattlesnake (E.R.) venom. 1 mg of egg (—) or beef (– – –) lecithin was the substrate. Results are shown as means ± S.E. of the mean.

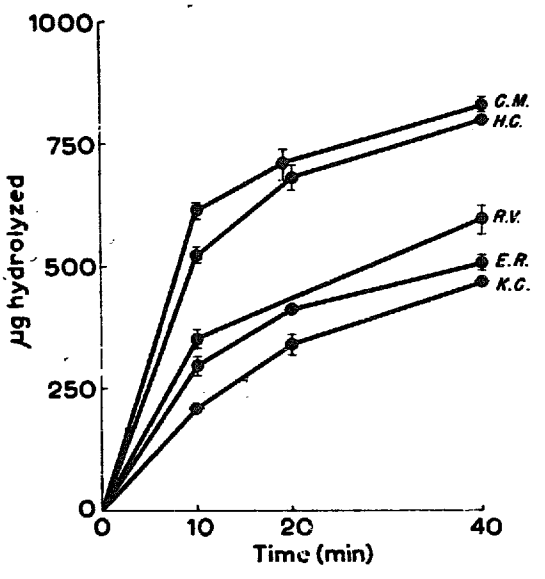


Fig. 5. Phospholipase A activities of 50 µg of cottonmouth-moccasin (C.M.), hooded cobra (H.C.), Russells viper (R.V.), Eastern diamond-back rattlesnake (E.R.) and king cobra (K.C.) venoms. 1 mg of beef lecithin was used as substrate. Results are shown as means ± S.E. of the mean.

A phospholipase A-rich fraction from cobra venom rendered curare active whereas a phospholipase A-poor fraction did not. Therefore the actual phospholipase A activities of the poor and rich fractions were checked and compared with hooded cobra venom from which these fractions were derived. The results are shown in Fig. 3.

It was reported by GOTTFRIED AND RAPPORT[16] that hooded cobra venom hydrolyzes egg lecithin at only a slightly faster rate than beef lecithin whereas Western diamondback rattlesnake venom hydrolyzes egg lecithin much better than beef lecithin. In addition, when they obtained purified plasmalogen from the beef lecithin the difference in hydrolyses of lecithin and plasmalogen by rattlesnake venom was even more marked. Since Eastern diamondback rattlesnake venom did not render curare active whereas hooded cobra and cottonmouth venom did it appeared that an action of the venom upon plasmalogen might be responsible for their ability to render curare active. Therefore the hydrolysis of egg and beef lecithin by cottonmouth and Eastern rattlesnake venom was tested. As seen in Fig. 4 cottonmouth venom hydrolyzes the two substrates at similar rates, whereas rattlesnake venom is significantly more effective in hydrolyzing egg than beef lecithin. When the hydrolysis of beef lecithin by 50 µg of various venoms was compared (Fig. 5), it was found that the two venoms most effective in rendering curare active[2] also hydrolyzed beef lecithin at the highest rates, but hydrolysis by the other three venoms did not correlate with their ability to render curare active.

We previously found that a compound described by ROSENTHAL AND GEYER as a phospholipase A and D inhibitor[20,21] did not antagonize the action of cottonmouth venom on the squid axon. We therefore checked to see if under our conditions this compound prevented the hydrolysis of egg or beef lecithin by 10 μg of cottonmouth venom. The venom did not hydrolyze the inhibitor. All absorbancy readings were corrected for the color due to the inhibitor alone. No inhibition of cottonmouth-venom phospholipase A activity by this compound was observed. The conditions are however not identical to those of ROSENTHAL AND GEYER[20,21] where an antagonism was found.

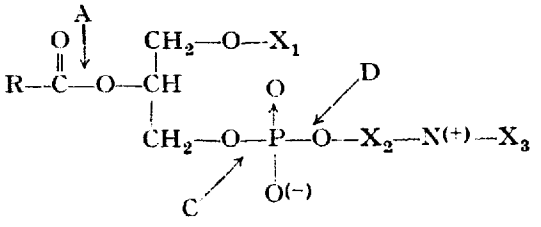
Table II shows that phosphodiesterase and phospholipase D rendered curare active, whereas phospholipase C, L-amino acid oxidase and Cobroxin were inactive. This might be due to a contamination with phospholipase A activity. As seen in Table III, phosphodiesterase contained considerable phospholipase A activity. Phospholipase C, Cobroxin and L-amino acid oxidase appeared to have low phospholipase A activity since they hydrolyzed only 10–20% of the egg lecithin. Almost all this hydrolysis occurred in the first 10 min.

DISCUSSION

The results reported in this study support the previous suggestions[1-4] that the phospholipase A of venoms is responsible for their ability to increase the permeability of the squid axon to normally impenetrable lipid-insoluble compounds such as ACh and curare. The increased permeability produced by the venoms allows ACh and curare to affect conduction by the mechanism suggested by NACHMANSOHN's theory[5,6]. The following enzymes, which are present in venoms, did not render the axon sensitive to the subsequent application of curare: L-amino acid oxidase (Table II), hyaluronidase[1] and proteolytic enzymes[1]. Venom phosphodiesterase which seemed to render curare active (Table II), was found to be contaminated with considerable phospholipase A activity (Table III). Cobroxin, a neurotoxic fraction of cobra venom with little or no enzymic activities, and used clinically for the relief of pain[25,26], did not render curare active (Table II). A fraction of cobra venom almost free of phospholipase A activity (Fig. 3) did not render curare active (Table II), whereas a fraction rich in phospholipase A activity (Fig. 3) rendered curare active when applied to the squid axon in relatively low concentrations (Table II). Heating cottonmouth venom at an alkaline pH destroyed its phospholipase A activity (Fig. 2) and also destroyed its ability to render curare active[2], whereas heating the venom at an acid pH had neither effect (Fig. 2 and ref. 2). The work of other investigators also indicates that phospholipase A is capable of disrupting membranes (see for example refs. 27–30). The source of phospholipase A in most of the studies was acid-heated solutions of venom. The effectiveness of the various snake venoms in rendering curare active was the same as their potencies in blocking conduction of the squid axon[2,3]. Alkaline heated venom solutions failed to affect conduction of the squid axon, whereas acid heated solutions were quite potent[2]. Phospholipase A may not, however, be the only venom component responsible for the direct effects on conduction since Cobroxin, a phospholipase A-poor fraction of cobra venom (Table I) and hyaluronidase[1] can block conduction of the squid axon although relatively high concentrations are required. Thus, it cannot be excluded, that other components contribute to the direct effect.

Assuming that phospholipase A is the active component of the venoms, it is of importance to know whether the venom effects are due to a direct action of phospholipase A on phospholipids or due to the liberation, by the action of phospholipase A on phosphatides, of lysophosphatides in the membrane. For example lysolecithin, a compound with powerful detergent properties, is formed by the hydrolysis of the fatty acid ester at the β-position of lecithin by the enzyme phospholipase A (see Scheme 1). Since the work of DELEZENNE AND LEDEBT[31] it is thought that hemolysis of red blood cells by venoms is due to the action of lysophosphatides. Both phospholipase A and lysolecithin cause demyelinating changes in the central nervous system[27] while lysolecithin and venom cause a release of glutamate–oxaloacetate transaminase from whole-cell preparations[32]. It was also suggested that block of conduction in lobster nerves by heated venom solutions may be due to the formation of lysophosphatides[33]. Other investigators[28–30,34], for various reasons, think the effects that they have observed are due to a direct action of phospholipase A and not due to a liberation of lysolecithin or other lysophosphatides. For example in two of these studies[29,34], phospholipase C had a similar effect as heated venom solutions which was taken as evidence that a lysophosphatide is not important in the action of phospholipase A (heated venom). The action of phospholipase C on phosphatides does not give rise to lysophosphatides (see Scheme 1).

It has not yet been ascertained whether phospholipase A is acting directly or whether lysophosphatides are responsible for increasing permeability to curare and ACh. We did find, however, that cottonmouth venom is less potent on the giant axons



	X_1	X_2	X_3
Phosphatidylcholine (lecithin)	O ‖ C—R O	$(CH_2)_2$	$(CH_3)_3$
Phosphatidylethanolamine	O ‖ C—R O	$(CH_2)_2$	H_3
Phosphatidylserine	O ‖ C—R	CH_2—CH | $COO^{(-)}$	H_3
Phosphatidalcholine (choline plasmalogen)	CH=CH—R	$(CH_2)_2$	$(CH_3)_3$
Phosphatidalethanolamine	CH=CH—R	$(CH_2)_2$	H_3
Phosphatidalserine	CH=CH—R	CH_2—CH | $COO^{(-)}$	H_3
Cephalin B	$(CH_2)_{17}CH_3$	$(CH_2)_2$	H_3

Scheme 1. Structure of several glycerophosphatides. A, C and D indicate the points of hydrolysis by phospholipase A, C and D, respectively. R = hydrocarbon chain.

of the stellar nerve where almost all small fibers were removed than on axons in which only part of the fibers were removed[2]. A likely explanation for this observation appears to be that in the closely dissected axons there is less substrate on which phospholipase A can act and therefore less lysophosphatides are formed. This is supported by the fact that phospholipase C has no direct effect on the squid axon (Table I) nor does it render curare active (Table II). If disruption of glycerophosphatides were the mechanism of action of phospholipase A, then phospholipase C should be effective also. Attempts were made to demonstrate the production by cottonmouth venom of a compound with potent effects on electrical activity. The venom was incubated with squid axons and with egg and beef lecithin, but no compound was obtained from the incubation mixture with potent effects on electrical activity. However, these negative results obviously do not exclude the possibility that such compounds may be formed.

Lecithin is probably not the phospholipid with which venom phospholipase A is reacting and which renders axons sensitive to curare since cottonmouth venom has only slightly greater phospholipase A activity than Eastern diamondback rattlesnake venom when egg lecithin is used as substrate (Fig. 4), while it is most effective in rendering curare active. In contrast, rattlesnake venom is completely inactive[2]. It has been reported that Western diamondback rattlesnake venom hydrolyzes phosphatidalcholine (choline plasmalogen) at a lower rate than phosphatidylcholine (lecithin), whereas little difference was observed in the rates of reaction of these two substrates with cobra venom[16]. Quantitatively, however, the difference in reported reaction rates[16] does not appear sufficient to account for the marked difference in the activity of venoms described here. We attempted to determine whether the ability of various venoms to render curare active[2] correlated with their ability to hydrolyze beef lecithin which contains about 60 % phosphatidalcholine (Fig. 5). Until isolated plasmalogen substrates are available, however, it will not be possible to reach any conclusions on the basis of these types of studies. Even with purified substrates it appears that the observed phospholipase A activity of venoms *in vitro* may be lower than their activity in the membrane, since according to the present data and previous reports[22] purified phospholipids are split at a lower rate than crude phospholipids. This may be due to the specific nature of the combination of phospholipid and protein in the crude substrate preparation[22], or it may be because purified substrates usually have saturated fatty acids at the β-position whereas the natural substrates contain mainly the unsaturated fatty acids as was found with choline plasmalogen[16].

The nervous system contains many phospholipids in addition to lecithin and choline plasmalogen (phosphatidalcholine); for example, phosphatidylethanolamine, phosphatidylserine, phosphatidalethanolamine, phosphatidalserine, cephalin B, inositol phosphatides and sphingomyelins (see for example refs. 35–41). The concentration of phosphatidalcholine is actually much less than the concentration of phosphatidalethanolamine[41,42]. Lecithin is not even a component of the myelin lipids whereas plasmalogens, inositol phosphatides and sphingomyelins are[38,43,44]. Quantitatively plasmalogens are one of the most important of the phosphatides found in white matter[37,45]. Different rates of hydrolysis of one or a mixture of these phosphatides may explain the marked difference in the abilities of the various venoms to render curare active.

In addition to the possibility that the phospholipase A in cottonmouth and rattlesnake venom have different specificities toward phospholipid substrates it is also possible that the enzymes have different physical properties which may explain the marked difference in their potencies. GOTTFRIED AND RAPPORT point out in discussing the hydrolysis of egg and beef-heart lecithin by cobra and rattlesnake venom that it can no longer be assumed that a single enzyme in each venom is responsible for the hydrolysis of both substrates[16]. Recent work indicates the presence in certain venoms of more than one phospholipase A, each with different physical properties[46,47].

If the venoms render curare active by liberating a lysophosphatide then lysolecithin on the basis of the above discussion is not likely to be the active lysophosphatide formed. Lysolecithin is a constituent of many tissues[48–51]. Lysolecithin was relatively weak in its direct effects on the squid axon (Table I) and did not render curare active (Table II). However, the external application of a lysophosphatide may not give results equivalent to its effects when formed within a membrane. In addition, lecithin present in the axonal preparation may have prevented the action of lysolecithin since it is known that lecithin decreases the detergent properties of lysolecithin[52,53]. Lysophosphatidylserine which has about the same hemolytic activity as lysolecithin[54] or lysophosphatidylethanolamine which has about twice the hemolytic activity of lysolecithin[55] or a lysoplasmalogen is more likely to be an active product of phospholipase A action in the effects observed than is lysolecithin.

Although phospholipase C and D are not present in venoms the effects of these two compounds on the squid axon are interesting. Neither of them had any direct effects on the action potential (Table I), however, phospholipase D even in relatively low amounts rendered curare active in contrast to phospholipase C (Table II). The block of conduction by curare following phospholipase D could so far not be reversed. Hydrolysis of phosphatides by phospholipase D removes a cationic nitrogen group. One may speculate that the cationic nitrogens of curare may be attracted to the negatively charged phosphates. The curare molecules may pass from one phosphate to another, and finally penetrate to the active sites of the neuronal membrane and affect electrical activity. The concentration gradient of curare and its binding might be such that with washing it would be difficult to remove enough of the curare to reverse its effects on electrical activity. Hydrolysis of phosphatides by phospholipase C removes the phosphate grouping as well as the cationic nitrogen leaving an uncharged diglyceride which would not be expected to bind curare.

ACKNOWLEDGEMENTS

We thank Dr. D. NACHMANSOHN for interest shown throughout the course of these studies and for helpful advice in the preparation of the manuscript. We are also grateful to the Marine Biological Laboratories, Woods Hole, Mass. for the facilities made available to us.

This work was supported by the Division of Research Grants and Fellowships, U.S. Public Health Service, Grants No. 2B-5216 and No. NB-03304, by the National Science Foundation Grant No. 12901, and by a Gift from the Muscular Dystrophy Associations of America, Inc. One of the authors (P.R.) was supported by U.S. Public Health Service Training Grant BT-579.

REFERENCES

1 P. ROSENBERG AND S. EHRENPREIS, *Biochem. Pharmacol.*, 8 (1961) 192.
2 P. ROSENBERG AND T. R. PODLESKI, *J. Pharmacol. Exptl. Therap.*, 137 (1962) 249.
3 P. ROSENBERG AND T. R. PODLESKI, *Biochim. Biophys. Acta*, 75 (1963) 104.
4 P. ROSENBERG AND F. C. G. HOSKIN, *J. Gen. Physiol.*, 46 (1963) 1065.
5 D. NACHMANSOHN, *Chemical and Molecular Basis of Nerve Activity*, Academic Press, New York, 1959.
6 D. NACHMANSOHN, *Science*, 134 (1961) 1962.
7 E. A. ZELLER, *Advan. Enzymol.*, 8 (1948) 458.
8 E. A. ZELLER, in J. B. SUMNER AND K. MYRBÄCK, *The Enzymes*, Vol. 1, Part 2, Academic Press, New York, 1951, p. 986.
9 E. E. BUCKLEY AND N. PORGES, *Venoms*, AAAS, Washington, 1956.
10 C. C. YANG, C. J. CHEN AND C. C. SU, *J. Biochem. (Tokyo)*, 46 (1959) 1201.
11 B. M. BRAGANCA AND I. ARAVINDAKSHAN, in K. A. C. ELLIOTT, I. H. PAGE AND J. H. QUASTEL, *Neurochemistry*, C. C. Thomas, Springfield, 2nd Ed., 1962, p. 840.
12 B. N. GHOSH, P. K. DUTT AND D. K. CHOWDHURY, *J. Indian Chem. Soc.*, 16 (1939) 75.
13 E. A. ZELLER, *Experientia*, 3 (1947) 375.
14 E. A. ZELLER, *Helv. Chim. Acta*, 32 (1949) 94.
15 W. L. MAGEE AND R. H. S. THOMPSON, *Biochem. J.*, 77 (1960) 526.
16 E. L. GOTTFRIED AND M. M. RAPPORT, *J. Biol. Chem.*, 237 (1962) 329.
17 J. M. R. BEVERIDGE AND S. E. JOHNSON, *Can. J. Res., Sect. E*, 27 (1949) 159.
18 D. WELLNER AND A. MEISTER, *J. Biol. Chem.*, 235 (1960) 2013.
19 E. J. WILLIAMS, S. C. SUNG AND M. LASKOWSKI, SR., *J. Biol. Chem.*, 236 (1961) 1130.
20 A. F. ROSENTHAL AND R. P. GEYER, *J. Biol. Chem.*, 235 (1960) 2202.
21 A. F. ROSENTHAL AND R. P. GEYER, *Arch. Biochem. Biophys.*, 96 (1962) 240.
22 E. CONDREA, A. DE VRIES AND J. MAGER, *Biochim. Biophys. Acta*, 58 (1962) 389.
23 A. HUGHES, *Biochem. J.*, 29 (1935) 437.
24 B. M. BRAGANCA AND J. H. QUASTEL, *Nature*, 169 (1952) 695.
25 D. I. MACHT, *Trans. Am. Therap. Soc.*, 40 (1940) 62.
26 R. G. HILLS AND W. M. FIROR, *Am. Surgeon*, 18 (1952) 875.
27 L. R. MORRISON AND P. C. ZAMECNIK, *Arch. Neurol. Psychiat. (Chicago)*, 63 (1950) 367.
28 A. P. NYGAARD, M. U. DIANZANI AND G. F. BAHR, *Exptl. Cell Res.*, 6 (1954) 453.
29 E. PETRUSHKA, J. H. QUASTEL AND P. G. SCHOLEFIELD, *Can. J. Biochem Physiol.*, 37 (1959) 975.
30 I. ARAVINDAKSHAN AND B. M. BRAGANCA, *Biochem. J.*, 79 (1961) 84.
31 C. DELEZENNE AND S. LEDEBT, *Compt. Rend.*, 155 (1912) 1101.
32 B. MCARDLE, R. H. S. THOMPSON AND G. R. WEBSTER, *J. Neurochem.*, 5 (1960) 135.
33 J. M. TOBIAS, *J. Cellular Comp. Physiol.*, 46 (1955) 183.
34 B. S. MELDRUM AND R. H. S. THOMPSON, *Guy's Hosp. Repts.*, 111 (1962) 87.
35 R. J. ROSSITER in K. A. C. ELLIOTT, I. H. PAGE AND J. H. QUASTEL, *Neurochemistry*, C. C. Thomas, Springfield, 2nd Ed., 1962, p. 10.
36 M. M. RAPPORT AND W. T. NORTON, *Ann. Rev. Biochem.*, 31 (1962) 103.
37 G. R. WEBSTER, *Biochim. Biophys. Acta*, 44 (1960) 109.
38 G. BRANTE, *Acta Physiol. Scand. Suppl.* 63, 18 (1949) 1.
39 A. C. JOHNSON, A. R. MCNABB AND R. J. ROSSITER, *Biochem. J.*, 45 (1949) 500.
40 R. E. MCCAMEN AND E. ROBINS, *J. Neurochem.*, 5 (1959) 18.
41 M. H. HACK, A. E. GUSSIN AND M. E. LOWE, *Comp. Biochem. Physiol.*, 5 (1962) 217.
42 M. M. RAPPORT AND N. F. ALONZO, *J. Biol. Chem.*, 235 (1960) 1953.
43 A. C. JOHNSON, A. R. MCNABB AND R. J. ROSSITER, *Arch. Neurol. Psychiat. (Chicago)*, 64 (1950) 105.
44 R. J. ROSSITER, in K. A. C. ELLIOTT, I. H. PAGE AND J. H. QUASTEL, *Neurochemistry*, C. C. Thomas, Springfield, 2nd Ed., 1962, p. 870.
45 M. M. RAPPORT AND B. LERNER, *Biochim. Biophys. Acta*, 33 (1959) 319.
46 H. M. DOERY AND J. E. PEARSON, *Biochem. J.*, 78 (1961) 820.
47 K. SAITO AND D. J. HANAHAN, *Biochemistry*, 1 (1962) 521.
48 G. B. PHILLIPS, *Proc. Soc. Exptl. Biol. Med.*, 100 (1959) 19.
49 R. NIEMIRO AND J. PRZYJEMSKI, *Acta Biochim. Polon.*, 8 (1961) 449.
50 G. R. WEBSTER AND R. H. S. THOMPSON, *Biochim. Biophys. Acta*, 63 (1962) 38.
51 N. M. PAPADOPOULOS, W. CEVALLOS AND W. C. HESS, *Arch. Neurol.*, 3 (1960) 677.
52 N. ROBINSON, *J. Pharm. Pharmacol.*, 12 (1960) 609.
53 E. GJONE, *Scand. J. Clin. Lab. Invest.*, 13 (1961) 369.
54 L. RATHBONE, W. L. MAGEE AND R. H. S. THOMPSON, *Biochem. J.*, 83 (1962) 498.
55 M. MATSUMOTO, *J. Biochem. (Tokyo)*, 49 (1961) 32.