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ABILITY OF VENOMS TO RENDER SQUID AXONS SENSITIVE TO CURARE AND ACETYLCHOLINE

PHILIP ROSENBERG AND T. R. PODLESKI

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

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SUMMARY

1. Cottonmouth-moccasin venom is more potent than copperhead moccasin, fer-de-lance or Western diamondback rattlesnake venom in rendering (+)-tubocurarine (curare) capable of affecting conduction of the squid giant axon.

2. Bee venom is more potent than the snake venoms. It irreversibly blocks axonal conduction in a concentration of 10 μg/ml. However, in lower concentrations (1–2 μg/ml) it does not render curare effective.

3. Following exposure to cottonmouth venom, $8.8 \cdot 10^{-4}$ M acetylcholine markedly decreased axonal electrical activity.

4. Physostigmine was antagonistic to the action of acetylcholine. An explanation for this apparently surprising effect is offered.

5. Cottonmouth venom also rendered the following compounds active: decamethonium, 2-pyridine aldoxime methiodide and 2-benzoylpyridine oxime. The potency of atropine and ethanol was increased by cottonmouth venom.

6. Carbamylcholine in $2.8 \cdot 10^{-2}$ M did not significantly affect conduction after pretreatment with cottonmouth venom while $1 \cdot 10^{-2}$ M nicotine had a weak effect.

INTRODUCTION

It had for many years been thought that the action of ACh, (+)-tubocurarine (curare) and other quaternary nitrogen compounds was limited to certain junctional regions. The inability of these compounds to affect axonal conduction was taken as evidence that ACh could not have an essential role in nerve conduction as described by NACHMANSOHN[1,2]. He and his associates, however, demonstrated the presence of barriers in the squid axon which prevent lipid-insoluble compounds such as ACh and curare from reaching the active neuronal membrane[3,4]. In preparations in which these barriers are not as great, ACh and curare do affect conduction. DETTBARN showed that curare rapidly and reversibly blocked electrical activity at Ranvier nodes of a single frog sciatic nerve fiber[5]. An effect of ACh upon conduction in the C-fibers of the desheathed vagus nerve of the rabbit was observed by ARMETT AND RITCHIE[6].

Abbreviations: ACh, acetylcholine; 2-PAM, 2-pyridine aldoxime methiodide; 2-benzoyl-PAM, 2-benzoylpyridine oxime.

Recently it was found that ACh affects electrical activity of nerve fibers from the walking legs of lobsters[7].

If ACh is inactive on other neuronal preparations because of permeability barriers it appeared possible that by chemical treatment one could decrease these barriers and demonstrate an action of lipid-insoluble compounds expected to interact with the ACh system. WALSH AND DEAL exposed frog sciatic nerves to a detergent and then observed reversible effects on conduction by ACh, curare etc.[8]. ROSENBERG and co-workers observed that following exposure of the squid axon to concentrations of certain venoms which had no effect of their own on conduction, curare, ACh and other lipid-insoluble compounds blocked conduction whereas on untreated axons they were inert[9,10]. It was also shown by ROSENBERG AND HOSKIN that on the untreated axon [^{14}C]ACh and curare do not penetrate into the axoplasm, whereas following cottonmouth-moccasin venom pretreatment significant amounts of these compounds are found in the axoplasm[11].

Four other venoms have now been studied in addition to cottonmouth-moccasin venom which previously was found most effective in rendering ACh and curare active[10]. The action of ACh and curare following exposure to cottonmouth venom has, moreover, been investigated in more detail with both extra- and intracellular electrodes. The effects on venom-pretreated squid axons of several additional compounds were studied.

METHODS AND MATERIALS

All solutions for application to the squid (*Loligo pealii*) axon were prepared in filtered sea water buffered at pH 7.6–7.8 with 1 mM Tris. This concentration of Tris has no effect on spike height over a period of several hours[9]. The temperature in all experiments was 18–22°. Unless otherwise specified all giant axons of the squid were dissected in the manner previously described[9], that is the majority but not all of the small adhering nerve fibers were removed. The methods used for recording of electrical activity are similar to those used previously[10], except that with intracellular recordings, a Varian paper recorder was occasionally used to monitor the resting potentials. When a block of conduction was noted with intracellular electrodes the axon was placed on external electrodes, and the extent of the blocked area was determined. When tested with extracellular electrodes, isolated control axons maintained their original spike heights $\pm$ 10% for at least 4 h, which was the longest period of any experiment. The axons used in this and a following study[12] had a mean initial action potential of 16.1 $\pm$ 0.1 mV, with a range from 7 to 40 mV (295 axons). With this method of recording electrical activity, decreases in spike height of 10% or less are not significant.

To determine whether a solution of venom rendered an axon sensitive to the subsequent application of ACh, curare etc. we used a similar procedure as previously described[10]. The axons were usually exposed to the venom solution for 30 min after which they were bathed in normal sea water for 10–15 min and then the compound under investigation was applied for 30 or 60 min unless block of electrical activity occurred earlier. To check reversibility the experimental solutions were replaced with normal sea water and electrical activity was checked for 20–40 min. The method used for calculating the percentage reversibility has been described[10]. All results in

the tables are recorded as mean ± S.E. (standard error or standard deviation of the mean).

$$\text{S.E.} = \sqrt{\frac{n\Sigma x^2 - (\Sigma x)^2}{n^2(n-1)}}$$

where x is an individual observation and n is the number of observations.

Snake venoms with their identifying lot numbers listed below were purchased from Ross Allen Reptile Institute, Silver Springs, Fla. All were lyophilized preparations except for *Bothrops atrox* which was dehydrated. *Agkistrodon p. piscivorus* (cottonmouth moccasin) 6-29-61; *Agkistrodon contortrix mokeson* (copperhead moccasin) 10-26-61; *Bothrops atrox* (fer-de-lance) 8–10-61; *Crotalus atrox* (Western diamondback rattlesnake) 5-28-61. Bee venom was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Crystalline (+)-tubocurarine chloride was purchased from K and K Laboratories, New York, N.Y. Solutions of egg and beef lecithin in absolute ethanol were purchased from Sylvana Chemical Co., Orange, N.J. Egg lecithin (Control No. EL-30x) had 36.6 mg of lecithin per ml of ethanol, while beef lecithin (L-80) nad 38.3 mg of phospholipids per ml.

RESULTS

Extracellular recordings

The direct effects on electrical activity of several venoms are shown in Table I. All the venom effects were irreversible which agrees with earlier results[10]. The data recorded for 15 μg/ml cottonmouth venom are in excellent agreement with previous results[10] and include those experiments in which the venom was used as a pretreatment (Tables II–IV).

We tested the effects of various quaternary ammonium compounds on the untreated axon. We confirmed our previous observation that on the untreated

TABLE I

THE DIRECT EFFECTS OF SEVERAL VENOMS ON THE ACTION POTENTIALS OF THE GIANT AXON OF SQUID

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

Venom	*Concn. (μg/ml)*	*No. of expts.*	*Exposure (min)*	*% decrease*	*% reversibility*
Cottonmouth moccasin	15	87	30 ± 0	6 ± 0.1	—
	50	14	32 ± 2	100 ± 0	0
Copperhead moccasin	250	3	55 ± 15	8 ± 5	—
	1000	2	10 ± 0	100 ± 0	—
Fer-de-lance	100	2	50 ± 0	0 ± 0	—
	500	2	55 ± 5	100 ± 0	0
	1000	2	43 ± 18	64 ± 36	0
Western rattlesnake	100	3	60 ± 0	9 ± 6	—
	200	2	50 ± 0	100 ± 0	0
	1000	2	10 ± 0	100 ± 0	0
Bee	2	6	26 ± 6	86 ± 14	0
	10	5	11 ± 2	100 ± 0	0

TABLE II

EFFECT OF CURARE ON THE ACTION POTENTIAL OF THE GIANT AXON OF SQUID FOLLOWING PRETREATMENT WITH VARIOUS VENOMS FOR 30 min (2 μg/ml OF BEE VENOM, 20 min)

Curare (5.6 mM) does not affect untreated axons. The results are given as means ± S.E. of mean.

Venom pretreatment	*Concn. (μg/ml)*	*No. of expts.*	*Curare (1.4 mM)*		*% reversibility*
			Exposure (min)	*% decrease*	
Cottonmouth moccasin	15	4	9 ± 1	93 ± 8	65 ± 10
Copperhead moccasin	200	2	30 ± 0	44 ± 24	50 ± 0
Fer-de-lance	200	2	30 ± 0	40 ± 24	68 ± 18
Western rattlesnake	150	4	30 ± 0	32 ± 13	—
Bee	1	6	40 ± 6	16 ± 5	—
	2	2	30 ± 0	11 ± 11	—

TABLE III

EFFECT OF ACh ALONE AND COMBINED WITH PHYSOSTIGMINE, CURARE OR ATROPINE ON ELECTRICAL ACTIVITY AFTER PRETREATMENT OF SQUID GIANT AXON WITH 15 μg/ml COTTONMOUTH-MOCCASIN VENOM FOR 30 min

The results are given as means ± S.E. of the mean.

Compound	*Concn. (M)*	*No. of expts.*	*Exposure (min)*	*Action potential (% decrease)*	*% reversibility*
ACh	$8.8 \cdot 10^{-4}$	8	60 ± 0	58 ± 10	13 ± 8
	$2.2 \cdot 10^{-4}$	5*	60 ± 0	30 ± 13	22 ± 4
ACh plus physostigmine	$4.4 \cdot 10^{-2} + 2.4 \cdot 10^{-4}$	4	60 ± 0	51 ± 28	—
	$8.8 \cdot 10^{-4} + 2.4 \cdot 10^{-4}$	8	60 ± 0	6 ± 6	—
	$8.8 \cdot 10^{-4} + 4.8 \cdot 10^{-5}$	5	60 ± 0	12 ± 2	—
	$8.8 \cdot 10^{-4} + 2.4 \cdot 10^{-6}$	2	60 ± 0	43 ± 3	—
ACh plus curare	$4.4 \cdot 10^{-3} + 1.4 \cdot 10^{-3}$	3	20 ± 0	90 ± 10	64 ± 8
	$4.4 \cdot 10^{-3} + 1.4 \cdot 10^{-4}$	3	30 ± 0	72 ± 14	—
	$4.4 \cdot 10^{-4} + 1.4 \cdot 10^{-3}$	2	25 ± 5	67 ± 20	100 ± 0
ACh plus atropine	$4.4 \cdot 10^{-4} + 5.0 \cdot 10^{-5}$	4	45 ± 9	67 ± 19	0 ± 0

* In these 5 experiments the pretreatment was 25 μg/ml cottonmouth venom.

axon these compounds are inert[9,10]. The following compounds when applied to the squid axon for 60 min in the concentrations (M) listed caused less than a 10% decrease in spike height. The number of experiments are indicated in parenthesis. Choline, $7.2 \cdot 10^{-2}$ (2); dimethylcurare, $1.1 \cdot 10^{-3}$ (4); curare, $1.4 \cdot 10^{-3}$ (2) and $5.6 \cdot 10^{-3}$ (2); decamethonium, $2.4 \cdot 10^{-2}$ (2); ACh, $4.4 \cdot 10^{-2}$ plus physostigmine, $2.4 \cdot 10^{-4}$ (4); 2-PAM, $1 \cdot 10^{-2}$ (3); 2-benzoyl-PAM, $1 \cdot 10^{-2}$ (3). $5 \cdot 10^{-4}$ M atropine sulfate decreased the spike height about 25% in 30 min (2) while $2 \cdot 10^{-4}$ M tetracaine reversibly blocked conduction in 15 min (2). 1 mM nicotine (2) had no effect on spike height in 30 min. Exposure to $1 \cdot 10^{-2}$ M nicotine for 60 min (9) decreased the spike height about 20%. The pH of the alkaloidal nicotine solution used in the above experiments was adjusted to about 8.0 with HCl.

TABLE IV

EFFECT OF SEVERAL COMPOUNDS ON ELECTRICAL ACTIVITY OF SQUID AXON FOLLOWING PRETREATMENT WITH 15 μg/ml COTTONMOUTH-MOCCASIN VENOM FOR 30 min

The results are given as means ± S.E. of the mean.

Compound	*Concn. (M)*	*No. of expts.*	*Exposure (min)*	*Action potential (% decrease)*	*% reversibility*
Decamethonium	$2.4 \cdot 10^{-2}$	7	8 ± 2	100 ± 0	0
	$2.4 \cdot 10^{-3}$	5	46 ± 7	35 ± 17	—
Carbamylcholine	$2.8 \cdot 10^{-2}$	6	60 ± 0	13 ± 2	—
	$5.6 \cdot 10^{-3}$	2	60 ± 0	9 ± 9	—
Atropine	$2.0 \cdot 10^{-4}$	3	20 ± 6	67 ± 20	72 ± 18
	$1.0 \cdot 10^{-4}$	2	30 ± 0	13 ± 4	—
2-PAM	$1.0 \cdot 10^{-2}$	4	55 ± 3	49 ± 23	0
2-Benzoyl-PAM	$1.0 \cdot 10^{-2}$	4	58 ± 3	58 ± 25	0
Nicotine	$1.0 \cdot 10^{-2}$	10	30 ± 0	27 ± 5	90 ± 10

Table II shows the results obtained when the ability of the venoms to render the squid axon sensitive to the blocking action of curare was tested. During pretreatment the venoms caused less than a 10% decrease in spike height except for 2 μg/ml of bee venom which caused about a 20% decrease. According to previous data on the untreated squid axon 5.6 mM curare was inactive. As in previous studies[10] cottonmouth venom was again the most effective pretreatment.

It was previously observed[10] that following cottonmouth venom, 4.4 mM ACh affected conduction but 0.44 mM did not. It also appeared that physostigmine antagonized the action of ACh. We explored the action of ACh following venom in more detail, and also tested whether in addition to physostigmine, curare and atropine also antagonize the action of ACh. The results are shown in Table III. In those experiments in which ACh was combined with physostigmine, the inhibitor alone, in the same concentration as when combined with ACh, was applied to the axon for 10 or 15 min immediately prior to the application of the combination. During this time physostigmine caused less than a 10% decrease in spike height. Although the average reversibility of the ACh effect was quite poor, 2 of the 8 experiments with $8.8 \cdot 10^{-4}$ M ACh showed 50% reversibility in 25 min.

Cobra-venom pretreatment did not render the squid axon sensitive to decamethonium or carbamylcholine[9]. These and several other compounds were tested following cottonmouth-venom pretreatment (Table IV). All of these compounds, except for atropine are inactive on the untreated axon. As described previously $5 \cdot 10^{-4}$ M atropine on the untreated axon caused about a 25% decrease in spike height in 30 min, while 1.5–6.0 mM atropine is required to decrease spike height 50% in 10–30 min[9]. Decamethonium, 2-PAM and 2-benzoyl-PAM were rendered active by the venom, while the potency of atropine sulfate was increased.

When 1 mg/ml of egg or beef lecithin were mixed with 50 μg/ml of cottonmouth venom and applied to the axon reversible block of electrical activity occurred in 30–40 min (8 experiments) in contrast to the consistently irreversible effect of this venom when applied alone. 1.4 mM curare also blocked electrical activity after this

treatment (4 experiments). Preparations of lecithin dissolved in ethanol were used (see MATERIALS) and solutions in sea water of 1 mg/ml of these lecithins contained about 2.6% ethanol. Since on the untreated axon 1 mg/ml of these lecithins had no effect in 30 min on spike height[12], it appeared that 2.6% ethanol has little effect on the untreated axon. Even 3% ethanol had no effect on electrical activity in 60 min (9 experiments) while 5% ethanol caused about a 63% reversible decrease in spike height in 60 min (2 experiments), and 10% ethanol reversibly blocked electrical activity in 5 min (2 experiments). 1.4 mM curare following 3% or 10% ethanol pretreatment did not affect electrical activity in 30 min (4 experiments). Experiments such as that illustrated in Fig. 1 clearly demonstrated that the venom rendered the axon sensitive to a normally ineffective concentration of ethanol. Following treatment of the squid axon with 15 μg/ml cottonmouth venom for 30 min which had no effect on electrical activity, 3% ethanol reversibly blocked conduction in about 10 min (3 experiments) while 2% ethanol caused about a 20% decrease in spike height in 30 min (3 experiments). Following pretreatment with 15 μg/ml cottonmouth venom plus 3% ethanol, which reversibly blocked electrical activity in 60 min, 4.4 mM ACh caused a 54% decrease in spike height in 60 min, while 0.44 mM ACh was inactive (4 experiments). Following 50 μg/ml venom plus 3% ethanol, which reversibly blocked electrical activity in about 20 min (9 experiments), 0.44 mM ACh blocked conduction irreversibly in about 50 min (2 experiments), while 1.4 mM curare caused about a 40% decrease in spike height in 30 min (2 experiments). 50 μg/ml cottonmouth venom plus 1% ethanol blocked electrical activity in about 44 min (4 experiments). This block was always irreversible indicating that in these experiments the venom blocked electrical activity not the ethanol. In the above experiments when venom plus ethanol pretreatment caused block, sea water was immediately added and reversal usually occurred in 5 min or less.

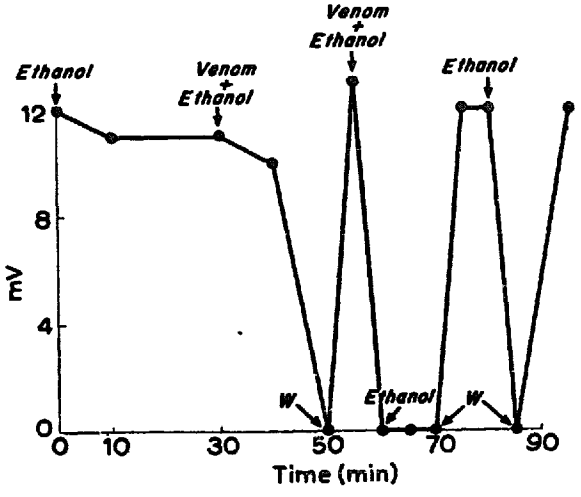

Fig. 1. Effect of 50 μg/ml cottonmouth-moccasin venom and 3% ethanol on the action potential of the squid giant axon. W indicates return to normal sea water.

Intracellular recordings

Control axons maintained their resting and action potentials with only small decreases over a period of about 3.5 h (Table V). In 6 experiments it was also observed that axons exposed to 15 μg/ml cottonmouth venom for 37 $\pm$ 4 min showed practically no change in resting or action potentials, and that when these axons were

returned to normal sea water for 66 ± 10 min there was no decrease in resting or action potential.

A few experiments were performed with cottonmouth venom on finely dissected axons in which almost all small fibers of the stellar nerve were removed, and on axons where many small nerve fibers were still attached to the giant axon (referred

TABLE V

EFFECT OF ACh AND CURARE ON RESTING POTENTIAL AND ACTION POTENTIAL OF CONTROL AXONS AND OF AXONS PRETREATED WITH 15 μg/ml COTTONMOUTH-MOCCASIN VENOM FOR ABOUT 30 min

The molar concentrations of curare and ACh are listed. R.P., resting potential. A.P., action potential. The results are given as means ± S.E. of the mean.

Treatment		*Exposure (min)*	*% decrease*		*No. of expts.*
Control	*After venom*		*R.P.*	*A.P.*	
—	—	214 ± 26	5 ± 5	8 ± 8	2
Curare ($1.4 \cdot 10^{-3}$)	—	113 ± 21	6 ± 2	6 ± 2	5
—	Curare ($1.4 \cdot 10^{-3}$)	29 ± 6	7 ± 2	48 ± 12*	13
ACh ($4.4 \cdot 10^{-2}$)	—	30 ± 0	5 ± 2	5 ± 2	5
ACh ($4.4 \cdot 10^{-2}$)	—	64 ± 3	12 ± 5	16 ± 5	5
—	ACh ($4.4 \cdot 10^{-2}$)	32 ± 4	16 ± 6	59 ± 11	7
—	ACh ($4.4 \cdot 10^{-3}$)	38 ± 8	6 ± 3	64 ± 22	5
—	ACh ($4.4 \cdot 10^{-4}$)	56 ± 4	4 ± 0	27 ± 17	2

* 5 out of the 13 axons were blocked by curare.

to as relatively crudely dissected axons). The observation, previously made with extracellular recordings[10], was confirmed, that is, the potency of this venom is greater on axons dissected in the usual manner (relatively crudely) than on finely dissected axons. All additional experiments using intracellular electrodes were therefore done with axons dissected relatively crudely.

The effects of ACh and curare on untreated axons and on axons previously exposed to 15 μg/ml cottonmouth venom are shown in Table V. The average exposure time to venom in the 32 experiments listed in Table V was 36 ± 2 min during which time the resting and action potentials decreased less than 3%. Fig. 2 shows the resting and action potentials associated with the action of ACh on cottonmouth-venom-treated axons. The effects of ACh were irreversible. In the experiments with extracellular electrodes the mean reversibility was also poor, only a few of the experiments showed reversibility. It is possible that the irreversibility observed with ACh was due to the techniques used in washing out the nerve chamber, which was quite different than that used in the extracellular experiments. Conduction block by $2 \cdot 10^{-4}$ M tetracaine was, however, found to be readily reversible.

Fig. 3 shows the effects of curare following treatment of axon with 15 μg/ml cottonmouth venom. In this experiment the effect of curare was reversible. In the majority of the experiments, however, no reversibility occurred, and replacement of the curare solution with normal sea water resulted in a slow depolarization. This

depolarization occurred only if curare had an effect on the action potential, whereas if curare was inactive replacement of the solution with normal sea water gave no modification of the electrical potentials.

Cottonmouth venom potentiated the action of ethanol. Fig. 4 shows an example of the effect of cottonmouth venom plus 3% ethanol.

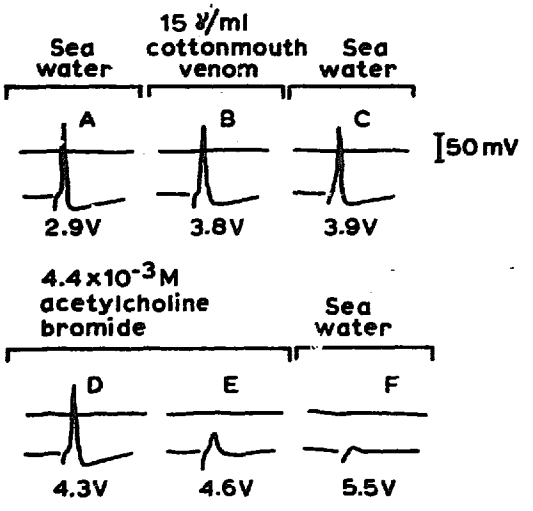

Fig. 2. Effect of ACh on the resting and action potential of the squid giant axon following exposure to cottonmouth venom. A, control; B, after exposure to 15 μg/ml venom for 30 min; C, 15 min after return to sea water; D, E, 15 and 25 min after exposure to 4.4 mM ACh; F, 30 min after return to sea water.

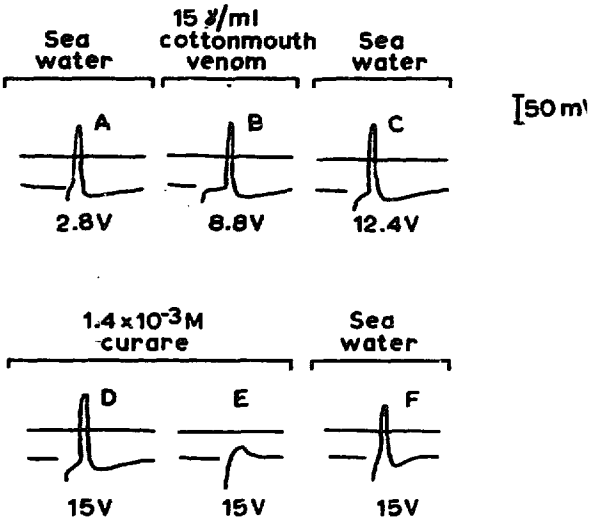

Fig. 3. Effect of curare on the resting and action potential of the squid giant axon following exposure to cottonmouth venom. A, control; B, after exposure to 15 μg/ml venom for 15 min; C, 15 min after return to sea water (stimulus voltage remained constant for this period); D, E, 4 and 8 min after exposure to 1.4 mM curare; F, 22 min after return to sea water.

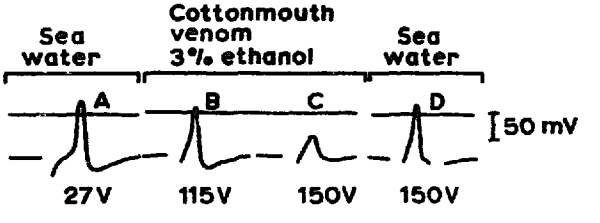

Fig. 4. Effect of 50 μg/ml cottonmouth venom plus 3% ethanol on the resting and action potential of the squid giant axon. A, control; B, C, 9 and 10 min after exposure to venom plus ethanol; D, 6 min after return to sea water.

DISCUSSION

Cottonmouth-moccasin venom has been found to be the most effective of nine different venoms in its ability to render curare capable of affecting electrical activity of the squid giant axon (Table II of this paper and ref. 10). The ability of the snake venoms to render curare active also correlates with their direct potencies on electrical activity of the squid giant axon, indicating that the same component of the venoms may be responsible for both effects. Studies by others of venom effects at the neuromuscular junction[13,14] have also shown cottonmouth venom to be more potent than rattlesnake venom, which in our studies was the least effective of the snake venoms. Likewise the relative effectiveness of cobra and rattlesnake venoms in our studies, and in causing demyelinating changes in the central nervous system[15,16] are similar. It is especially interesting that the hemolytic and hemaglutinen activities of several

snake venoms as found by MINTON[17] is in excellent agreement with their abilities to render curare active. For example, MINTON reports that Eastern diamondback rattlesnake venom has little or no hemolytic activity whereas Western rattlesnake venom is much more potent which is in agreement with our findings. Biochemical studies on the venom of the Western rattlesnake have been recently reported[18]. MINTON also found in his studies, as we found in ours, that copperhead moccasin is much less potent than cottonmouth-moccasin venom. The marked similarity between our results and his may indicate the same venom component is responsible for both effects being measured. The possible nature of this venom component, and its mechanism of action will be discussed in a succeeding paper[12].

An important factor in the action of these venoms is their ability to alter membrane permeability, which was known even before the work of DELEZENNE AND LEDEBT[19,20] on mechanism of hemolysis by venoms. More recently moccasin venom was found to increase the penetration of perfusion fluid through the frog atria[21] and the penetration of procaine into sciatic nerve[22]. It has recently been shown with the aid of radioactive ACh and curare that after exposure of the squid axon to cottonmouth venom they penetrate into the axoplasm, whereas rattlesnake venom which does not render the compound active also does not permit penetration[11]. Thus a direct evidence has been offered for the assumption that increased permeability is responsible for the effects of ACh and curare after venom treatment. The mere penetration of ACh and curare would not of course explain its effects on electrical activity. But in view of the presence of the entire ACh system in the squid axonal membrane and the functional interdependence in the electrical activity[1,2] the observations indicate that these compounds interact with an ACh receptor in the membrane and thereby alter electrical activity as suggested by NACHMANSOHN[1,2].

The exact nature of the permeability barriers and their alteration by the snake venoms is not known. The action of phospholipase A (EC 3.1.1.4) is probably one important factor in the process. Electron microscope studies in progress may offer some indication as to the site of venom action. But it is possible that minor chemical reactions such as removal or reorientation of phospholipid groups are responsible for permitting compounds to reach the normally protected active sites.

Bee venom was the most potent of any of the venoms tested in its direct effects on electrical activity (Table I), but in the lower concentrations tested (1–2 μg/ml) it did not render curare active (Table II), whereas in the higher concentrations (10 μg/ml) it blocked conduction by itself. It has been shown, however, that the concentration of bee venom which blocks electrical activity markedly increases the penetration of curare into the axoplasm of the squid axon, whereas the lower concentration which did not render curare active did not increase the penetration of curare[11]. It thus appears that the safety margin with bee venom may be much smaller than with cottonmouth venom, *i.e.*, that it is more difficult to find a concentration of bee venom which increases permeability without at the same time affecting electrical activity. This may be related to the composition of bee venom which is markedly different from that of the snake venoms. The only enzymes known to be present in bee venom are phospholipase A (EC 3.1.1.4) and hyaluronidase (EC 4.2.99.1)[23,24], in contrast to the much greater variety of enzymes in snake venoms. In addition a fraction can be isolated from bee venom which has no enzymic activity, but which is very toxic[24].

The effects of ACh on electrical activity of squid axon are a further indication that in view of the many permeability barriers surrounding the conducting membranes the external concentrations of compounds required to affect electrical activity have little meaning, as was so frequently emphasized by NACHMANSOHN[1]. On untreated axons or following cobra-venom pretreatment[9] ACh did not affect electrical activity, whereas following 15 μg/ml of cottonmouth venom we observed effects of 4.4 mM ACh[10], and have now obtained effects with concentrations as low as $8.8 \cdot 10^{-4}$ M (Table III). By increasing the concentration of cottonmouth venom to 25 μg/ml (using only those axons not directly affected by the venom) we have obtained an effect of ACh with the low concentration of $2.2 \cdot 10^{-4}$ M. It appears probable that with more elaborate experiments trying to render the pretreatment more effective, such as *e.g.* by isolating the active component of venoms, effects of ACh at still much lower concentrations would be obtained. It is pertinent, that in the present study the action of various additional quaternary ammonium ions was demonstrated after treatment, compounds which otherwise do not effect axonal conduction.

Physostigmine antagonizes the action of ACh on electrical activity after venom treatment. It was similarly observed by DETTBARN AND DAVIS[25] in the lobster axon that physostigmine usually antagonized the action of ACh. Physostigmine has a dual action; in lower concentrations it inhibits acetylcholinesterase (EC 3.1.1.7) and in higher concentrations it acts as a receptor inhibitor[1,26]. It would be expected therefore to act synergistically with ACh. One possible explanation for our results may be as follows: physostigmine is at the pH used, to a large part cationic. The pathways for cationic molecules even after venom treatment, are limited as many experiments clearly indicated that only a small fraction of the outside concentration penetrates to the active sites. The cationic molecules if present in sufficiently high concentrations may compete with ACh for these pathways and prevent ACh from reaching the active sites, but having a low affinity to the receptor may not be able in the concentration in which they penetrate to affect electrical activity. Thus the result would be an apparent antagonism. This view is open to experimental test and will be evaluated in future investigations. We did not observe any antagonism of the action of ACh by curare or atropine (Table III). It will require additional experiments, however, to determine if the concentrations selected or conditions of application were not optimal for observing an antagonism.

The ability of cottonmouth venom to increase the potency of ethanol, may be an important tool in further venom studies. Instead of arbitrarily applying venom for a fixed length of time to all axons, it may be more effective to apply a mixture of venom plus ethanol until electrical activity is blocked since with appropriate concentrations of ethanol this block is due to the ethanol and is reversible. Following reversible block by cottonmouth venom plus ethanol, curare and ACh did reversibly affect electrical activity. This method of pretreatment may be a more efficient guide for obtaining the optimal conditions in which compounds like curare, ACh etc. become effective. With the present method only a certain percentage of the axons are rendered sensitive to these agents[9,10].

With careful dissection of the giant axon and with the use of intracellular electrodes it was found that cottonmouth venom did not render squid axons sensitive to the action of curare to the extent that was observed with extracellular electrodes[10]. It was suggested that this was because finely dissected axons were used with the

intracellular recordings whereas relatively crudely dissected axons were used with the extracellular recordings. It was shown using extracellular electrodes that cottonmouth venom was less potent on finely dissected axons than on crudely dissected axons[10] and possible reasons for this were discussed. After confirming this observation using intracellular electrodes all further studies were performed using axons dissected in the usual manner, *i.e.* relatively crudely. Under these conditions curare had a greater effect on conduction after cottonmouth venom than was previously observed. We also confirmed with intracellular electrodes the marked effect of ACh on conduction following cottonmouth venom.

A 15–20% decrease in the resting potential by 1.4 mM curare was previously observed on finely dissected axons[10]. In the present study no such effect was found. Two major differences in the experimental conditions may account for this. The present experiments were performed at a lower temperature, and larger squids were available allowing sufficient lengths of axons to be obtained without dissecting a large portion of the fiber from beneath the mantle muscle. It may however be mentioned that HODGKIN AND KEYNES[27] observed following injection of small amounts of curare into axoplasm of squid a small depolarization, although no effect on conduction.

In the experiments with intracellular electrodes $4.4 \cdot 10^{-2}$ M ACh following venom had a somewhat greater effect on the resting potential in 30 min than was observed without venom pretreatment. However, 4.4 mM ACh following venom had a marked effect on the action potential but apparently no effect on the resting potential. This is in contrast to other axonal preparations in which effects of ACh on the action potential were paralleled by a concurrent effect on the resting potential[6,7,30]. This question requires further investigations.

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