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INCREASED CHOLINESTERASE ACTIVITY OF INTACT CELLS CAUSED BY SNAKE VENOMS*

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Abstract—Cottonmouth moccasin and Eastern diamondback rattlesnake venom increase the cholinesterase (ChE) activity observed in intact squid and lobster nerves and single electroplax. Cottonmouth was more effective than rattlesnake venom in lobster nerve and electroplax. Neither venom increased the activity of rabbit cerebral cortex slices or of a partially purified preparation of electroplax ChE. Both venoms are free of ChE activity. Strong permeability barriers are present in homogenized electroplax; they can be most effectively reduced by the use of the venoms. The venoms cause a reduction in the wet and dry weights of electroplax cells. They also depolarize and block electrical activity in concentrations of 50–100 μg/ml. Venoms may be useful in studies where it is necessary to measure the total ChE activity in intact cell preparations containing permeability barriers to the substrate normally employed in this enzyme assay.

IT IS difficult to determine the total cholinesterase (ChE) activity in intact nerve fibers. Substrates such as acetylcholine (ACh) and acetyl β-methylcholine (MeCh) are lipid insoluble; therefore they may not penetrate to all of the enzyme and hence do not permit measurement of total ChE activity. In attempting to overcome these difficulties several procedures have been tried. None of them, however, is entirely satisfactory. The use of a less specific but more lipid-soluble substrate,[1, 2] dimethylaminoethyl acetate for example, still does not exclude only a partial saturation of the enzyme with substrate and in addition raises the question of the type of esterase being assayed.

It appeared of interest to investigate whether the reduction of permeability barriers in the intact tissue might result in higher ChE activity by allowing ACh or MeCh to penetrate to most of the available enzyme. Venoms increase the permeability of various biological membranes.[3–6] It was recently demonstrated that, whereas on the untreated squid giant axon *d*-tubocurarein (curare) and ACh were inactive in altering the resting potential or conducted action potential, after the use of cottonmouth moccasin venom both agents reversibly block the propagated action potential and in some cases decrease the resting potential.[5, 7] This effect of the venom appears due to an increase in the permeability of the structures surrounding the axon, normally impervious for lipid-insoluble compounds.[6] More recently it was shown in the nerves from the walking

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legs of lobsters, where ACh acts without requiring any pretreatment,[8] that the use of cottonmouth venom reduces the concentration of ACh required.[9]

This paper describes studies of ChE activity in intact and homogenized tissues measured with and without exposure to venoms.

METHODS AND MATERIALS

The hindmost stellar nerve of the squid (*Loligo pealii*) was dissected as previously described.[5] The preparation consisted of the giant axon plus many small nerve fibers. The ends of the nerves were ligated, nerves weighed and placed for 20 min either in sea water containing 1 mM Tris, (pH 7·8), or placed in a solution of venoms in sea water. Each axon was then rinsed for 10 min in sea water and either incubated intact or homogenized by hand with a Tenbroek tissue grinder in sea water containing 0·1 M Tris (pH 7·7–7·9; temperature 19 to 21°). ACh bromide (5×10^{-3} M) was used as substrate. Cholinesterase activity was determined by the Hestrin colorimetric method.[10]

A procedure similar to that above was used with the other tissues except for the following modifications. With rabbit brain a mammalian medium containing 150 mM NaCl, 6 mM KCl, 3·3 mM $CaCl_2$, 1·5 mM $MgCl_2$, 10 mM glucose, and (in solutions used for enzyme assay) 0·1 M Tris was used. In the studies with isolated single electroplax from *Electrophorus electricus* a special Ringer's solution was used.[11] In every series of related experiments cells from a single eel were used, since variations in ChE from cell to cell of the same eel were less than variations between cells of different eels. In some of the experiments with electroplax 1×10^{-2} M MeCh was used as substrate rather than 5×10^{-3} M ACh. All slices of rabbit cerebral cortex were made by hand with a razor blade. In some experiments with electroplax, brain, and lobster nerves, the tissues were homogenized directly in the incubation media containing substrate and venom rather than preincubated for 30 min with venoms.

All results in the tables are presented as means $\pm$ standard deviations of the mean (standard error).[5]

The effect of the venoms on the directly and indirectly evoked action potentials of the electroplax were determined with intracellular KCl-filled microelectrodes of 3–10 MΩ resistance by the method of Higman and Bartels.[12]

Lyophilized venoms of *Agkistrodon p. piscivorus* (cottonmouth moccasin, lot 6-29-61) and *Crotalus adamanteus* (Eastern diamondback rattlesnake, lot 2-2-61) were purchased from Ross Allen Reptile Institute, Silver Springs, Fla. We are grateful to Dr. H. C. Lawler for supplying us with a partially purified preparation of eel acetylcholinesterase.[13]

RESULTS

It has been reported that only venoms of the Elapidae contain cholinesterase activity.[14, 15] In a series of 12 experiments using 1 mg cottonmouth or rattlesnake venom/ml and 5×10^{-3} M ACh as substrate we confirmed that these venoms are devoid of ChE activity.

The suspension of homogenized axons of the squid stellar nerve after pretreatment with cottonmouth or rattlesnake venom had about the same activity as the control suspension, indicating that these venoms in addition to lacking cholinesterase activity also lack anticholinesterase activity (see Table 1). The activity of a partially

purified enzyme preparation from the electric eel is also not significantly changed by the addition of the venoms, as is seen in Table 4.

Recent, as yet unpublished, experiments by Brzin, Dettbarn and Rosenberg, using a magnetic diver method, indicate that 96 to 98% of the enzyme activity in Table 1 is due to the small nerve fibers and only 2–4% of the total activity to the giant axon.

TABLE 1. CHOLINESTERASE ACTIVITIES OF HOMOGENIZED AND INTACT SQUID NERVES WITH AND WITHOUT COTTONMOUTH MOCCASIN (CM) OR RATTLESNAKE (RS) VENOM PRETREATMENT

Acetylcholine (ACh), 5×10^{-3} M, used as substrate. Results are presented as means ± S.D.M.

Venom	(μg/ml)	No. of experiments: Homogenized	No. of experiments: Intact	(μmoles ACh hyd./g/hr)
		17		54 ± 3
CM	500	4		58 ± 7
RS	1,000	3		51 ± 7
			17	44 ± 2
CM	15		9	52 ± 4
CM	100		9	55 ± 4
RS	100		8	58 ± 3
RS	1,000		8	54 ± 4

Intact nerves had significantly lower ($P < 0{\cdot}01$) enzyme activity than homogenized nerves (about 20% lower; Table 1). Enzyme activity of intact axons measured after exposure to either of the two venoms was significantly increased ($P < 0{\cdot}01$ to $P < 0{\cdot}05$) to about the same level observed with homogenized axons. A single squid stellar nerve was used for each experiment listed in the Table. The wet weight of the nerves based on 18 measurements was 34 ± 1 gm (mean ± standard deviation of the mean).

Both venoms markedly increased the observed ChE activities in homogenized and intact electroplax cells (Table 2). The wet weight of the cells based on 145 measurements was 37 ± 1 mg (mean ± standard deviation of the mean). In intact cells cottonmouth venom was more effective than rattlesnake venom. We tested the effect of varying the time of preincubation with venom from 15 to 60 min. The venom exerted its maximal effect within 15 min, although as noted in the Methods section all results in the tables are based upon a 30-min incubation with venom. The highest activities were observed when the cells were homogenized in the presence of venoms.

The electroplax cells are quite difficult to homogenize. Dense connective tissue remains even after the most vigorous homogenization, but, when this connective tissue was tested, no ChE activity was detectable. Fine strands of cellular tissue were often noted in the homogenized samples; therefore these samples were subjected to various treatments in an attempt to demonstrate the presence of barriers and their disruption.

Long periods of homogenization (B) or freezing and thawing of the cells (C) increased the observed enzyme activity about one third (Table 3). Filtering suspensions of homogenized cells through fine cheesecloth decreased the activity about one third,

TABLE 2. EFFECTS OF VENOMS ON CHOLINESTERASE ACTIVITY OF HOMOGENIZED AND INTACT ELECTROPLAX CELLS

Group A = homogenized cells, acetylcholine (ACh) as substrate; B = intact cells, ACh; C = homogenized cells, acetyl β-methylcholine (MeCh) as substrate; D = intact cells, MeCh. CM, RS = intact cells preincubated with cottonmouth or rattlesnake venom and enzyme activity of homogenized or intact cells measured in absence of venom; CM*, RS* = cells homogenized directly in assay media containing either venom.

Venom	(μg/ml)	No. of expts.	(μmoles hyd./cell/hr)
Group A			
		18	18·3 ± 0·9
CM	50	2	19·9 ± 2·8
CM	400	4	29·5 ± 0·3
CM	1,000	2	31·5 ± 1·5
CM*	50	3	44·0 ± 6·5
CM*	1,000	6	66·1 ± 3·8
RS	50	2	19·9 ± 3·8
RS	400	4	29·7 ± 1·8
RS	1,000	2	31·7 ± 0·6
RS*	50	3	28·1 ± 1·9
RS*	1,000	3	43·4 ± 3·6
Group B			
		12	3·2 ± 0·3
CM	50	3	5·0 ± 0·5
CM	400	6	10·3 ± 0·5
CM	1,000	3	19·7 ± 1·8
RS	50	3	4·0 ± 0·5
RS	400	6	7·1 ± 1·2
RS	1,000	3	8·7 ± 0·6
Group C			
		3	6·2 ± 0·2
CM	1,000	2	8·8 ± 2·4
RS	1,000	2	9·3 ± 0·8
Group D			
		3	1·7 ± 0·3
CM	1,000	3	7·1 ± 0·7
RS	1,000	3	4·1 ± 0·8

TABLE 3. CHOLINESTERASE ACTIVITY OF ELECTROPLAX CELLS

Treatment A = cells homogenized in usual manner (1 min); B = cells vigorously homogenized (10 min); C = cells 3 times rapidly frozen in acetone–dry ice mixture (2 min) and then thawed at room temperature (15 min). As noted below, some of these cells were then homogenized while other were placed unhomogenized in venom or control incubation mixtures. CM, RS, CM*, RS* as in Table 2. ACH used as substrate.

Treatment	Venom	(μg/ml)	No. of expts.	(μmole ACH hyd./cell/hr.)
		Homogenized		
A			6	22 ± 2
B			3	33 ± 1
C			3	33 ± 1
A	CM*	1,000	6	45 ± 3
B	CM*	1,000	3	47 ± 1
C	CM*	1,000	3	67 ± 1
C	RS*	1,000	3	48 ± 1
		Not homogenized		
C			3	10 ± 1
C	CM	1,000	3	61 ± 7
C	RS	1,000	3	15 ± 1

demonstrating the presence of gross particles in cells homogenized in the usual manner. The procedures, however, did not increase the activity of the homogenized tissues up to that observed in the presence of venom, indicating the presence of microscopic barriers present even in vigorously homogenized tissue. Cottonmouth venom increased the activity to about the same extent whether the cells were homogenized vigorously (B) or not (A). The venoms seemed most effective in increasing

TABLE 4. EFFECT OF VENOMS ON A PARTIALLY PURIFIED ACETYLCHOLINESTERASE PREPARATION OBTAINED FROM *Electrophorus electricus*

CM, RS = cottonmouth moccasin and rattlesnake venoms. ACh used as substrate.

Venom	(μg/ml)	No. of expts.	(μmoles ACh hyd./5 μg/hr)
CM		3	15·4 ± 1·5
CM	100	3	17·2 ± 0·3
CM	1,000	3	17·4 ± 0·7
RS	100	3	16·4 ± 0·6
RS	1,000	3	16·8 ± 0·2

TABLE 5. EFFECT OF COTTONMOUTH MOCCASIN (CM) AND RATTLESNAKE (RS) VENOM ON ELECTROPLAX CELL WEIGHTS

The wet weights of the cells were determined at the beginning of the experiment, after incubation in electroplax Ringer's with or without venom for 30 min (A), and after 1 hr in solution containing 0·1 M Tris used for determination of enzyme activity (B). The dry weight was determined after exposure to a temperature of 100°C for 18 hr.

Venom	(μg/ml)	No. of expts.	% Decrease in cell weight		Dry wt. × 100
			A	B	Original wt.
		27	0*		
		9	3 ± 2	15 ± 4	4·30 ± 0·33
CM	50	5	22 ± 4		
CM	200	5	46 ± 1		
CM	400	5	56 ± 3		
CM	1,000	8	57 ± 4		
CM	1,000	8	57 ± 5	85 ± 9	1·76 ± 0·06
RS	50	5	17 ± 2		
RS	200	5	41 ± 4		
RS	400	5	46 ± 1		
RS	1,000	8	52 ± 9	67 ± 5	2·91 ± 0·17

* Increase in cell weight of 2 ± 1%.

activity when the cells were first frozen and thawed (C). After freezing and thawing the cells, cottonmouth venom caused a much greater increase in the observed enzyme activity than did rattlesnake venom, confirming the findings reported in Table 2. Under comparable conditions the only procedure found in which the homogenized

and unhomogenized cells displayed equal and maximal activity was when the unhomogenized cells were first frozen, then thawed, and then treated with 1,000 μg of cottonmouth venom/ml (Table 3).

As seen in Table 4, neither venom significantly alters the activity of a soluble, partially purified preparation of acetylcholinesterase (AChE) ($P > 0{\cdot}10$) where any permeability barriers would be minimal.

A shrinking of the electroplax was noted after incubation with venom. The venoms even in relatively low amounts caused a reduction in wet and dry weights of the cells (Table 5).

Six experiments with intracellular electrodes indicated that both venoms, in a concentration of 50–100 μg/ml, block electrical activity, with depolarization observed especially with the higher concentrations. The effect of 50 μg cottonmouth moccasin venom/ml is shown in Fig. 1, where a small depolarization was observed.

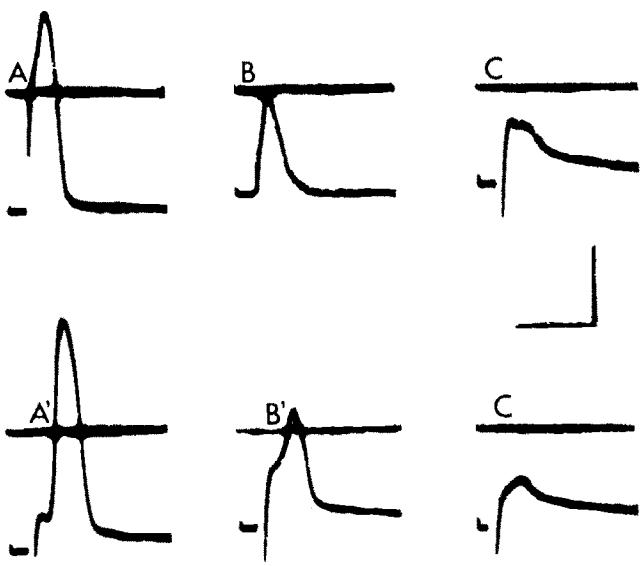

FIG. 1. Effect of cottonmouth moccasin venom on the electrical activity of the isolated single electroplax. A, B, C — Response to direct stimulation; A′, B′, C′ = response to indirect stimulation. AA′ = Control; BB′ and CC′ = 5 and 10 min after exposure to 50 μg venom/ml. Effects were irreversible. Calibration lines indicate 50 mV and 4 msec.

Neither venom had any effect on the ChE activity of homogenized or intact rabbit cerebral cortex slices (Table 6). Each incubation flask contained 35–45 mg (wet weight) of brain slices.

As seen in Table 7 both venoms increased the activity of intact lobster nerve, although neither altered that of the homogenized preparations. The lobster nerves were divided into equal segments of 25–30 mg (wet weight), and one segment was used per incubation flask. In several experiments the time of preincubation with venom was varied from 15 to 60 min. Maximal effects were obtained within 15 min, although the results in the table are based upon a 30-min incubation. Cottonmouth

venom is more effective than rattlesnake venom also in this preparation; it doubled the activity observed in intact lobster nerve, bringing it up to about 75% of that in the homogenized nerves.

Neither venom altered the weight or appearance of rabbit cerebral cortex slices or lobster nerves.

TABLE 6. EFFECTS OF VENOMS ON THE CHOLINESTERASE ACTIVITY OF HOMOGENIZED AND INTACT RABBIT CEREBRAL CORTEX SLICES

CM, RS. CM*, RS* as in Table 2. ACh used as substrate.

Venom	(μg/ml)	No. of experiments: Homogenized	No. of experiments: Intact	(μmoles ACh hyd./g/hr)
		12		260 ± 20
CM	1,000	9		254 ± 29
CM*	1,000	9		285 ± 22
RS	1,000	6		227 ± 18
RS*	1,000	6		256 ± 27
			12	138 ± 9
CM	1,000		9	152 ± 10
RS	1,000		6	156 ± 16

TABLE 7. EFFECTS OF VENOMS ON THE CHOLINESTERASE ACTIVITY OF HOMOGENIZED AND INTACT NERVES FROM THE WALKING LEG OF LOBSTER

CM, RS, CM*, RS* as in Table 2. ACh used as substrate.

Venom	(μg/ml)	No. of experiments: Homogenized	No. of experiments: Intact	(μmoles ACh hyd./g/hr)
		9		925 ± 51
CM	1,000	6		1,147 ± 121
CM*	1,000	6		886 ± 160
RS	1,000	3		924 ± 62
RS*	1,000	3		900 ± 68
			14	337 ± 20
CM	100		4	442 ± 20
CM	300		4	584 ± 53
CM	1,000		6	698 ± 36
RS	1,000		4	475 ± 45

DISCUSSION

Venoms, particularly their phospholipase A component, are known to increase permeability and disrupt various biological membranes.[3-7, 16] The data presented show that venoms cause a marked increase in ChE activity of intact axons of the squid stellar nerve, the fibers of the lobster walking-leg nerve, and the isolated eel electroplax. Since the venoms used have no cholinesterase activity, their effects are attributed to a greater accessability of ChE in the intact preparations. The squid stellar nerve is composed of the giant axon plus small nerve fibers. Since giant axons contain only 2–4% of the total ChE activity of the squid stellar nerve, the experiments reported

here would not indicate whether rattlesnake and cottonmouth venom increased the enzyme activity of the giant axon. The majority of the ChE is in the membrane of the giant axon[17, 18] and, since the surface area of the giant axon is much less than the total surface area of the small nerve fibers, it is not surprising that the majority of the ChE is in the small fibers. Rattlesnake venom is much less effective than cottonmouth venom in blocking conduction of the giant axon and in rendering it sensitive to curare.[5] Also in contrast to cottonmouth venom, it does not increase the permeability of the giant axon to ^{14}C-dimethyl curare.[6] One might suspect, therefore, that rattlesnake venom would not increase the accessibility of ChE in the intact giant axon. It would, however, require the use of a more sensitive method of measuring ChE activity to settle this point because of the relatively low enzyme activity of the giant axon. Since phospholipase A of the venoms appear responsible for their effects on the squid giant axon[16] as described above, and since it is known that venoms hydrolyse phospholipids at different rates,[19] it is possible that the phospholipid composition of the small nerve fibers is different from that of the giant axon. This could explain the ability of rattlesnake venom to increase the accessibility of ChE in the stellar nerve (mainly small fibers) and its inability to increase permeability of the squid giant axons.[6]

The increased permeability caused by the venoms in lobster nerve agrees with the finding that cottonmouth venom decreases the concentration of ACh required to affect electrical activity.[9]

Permeability barriers appear to be present in the intact rabbit cerebral cortex slices as is shown by the double ChE activity observed upon homogenization, but neither venom was able to increase the accessibility of the enzyme to ACh in these slices. If phospholipase A is the active component of the venoms in these studies, the results may indicate either an inability of phospholipase to reach the phospholipid sites in the membrane or the presence in the cortex of certain phospholipids or complexed phosphatides which are not attacked by these venom preparations.

An interesting and unexpected finding to us was the demonstration in homogenized electroplax of strong permeability barriers. They could be partially eliminated by longer periods of homogenization or by freezing and thawing of cells; however, the use of venoms either alone or after one of the above procedures appeared best.

The greater effectiveness of cottonmouth than rattlesnake venom in increasing permeability in intact electroplax and lobster nerve agrees with findings in other systems that rattlesnake venom is weak in its ability to disrupt membranes.[5, 6, 20–22] It is of interest that in homogenized electroplax both venoms were about equally effective in disrupting these permeability barriers, indicating perhaps an inability of rattle snake venom phospholipase A to penetrate to substrate sites in the membrane of intact preparations. It is known that venoms contain more than one phospholipase A, each with different physical properties.[23, 24]

Although venoms contain many enzymatic and nonenzymatic components, it appears probable that the increased accessibility of ChE observed in these studies is due to phospholipase A. Previous studies on the squid giant axon, using various venom components and selectively heating venom preparations, have indicated that phospholipase A is responsible for the increased permeability of this axon to ACh, curare, etc.[5, 6, 16] The same venom component is probably responsible for the effects observed in lobster nerve and electroplax.

The decrease in both wet and dry weight of electroplax cells exposed to venoms may be due to the action of various enzymes present in the venoms, in addition to phospholipase, such as protease, phosphodiesterase, hyaluronidase, etc.

Venoms may be useful in future studies where it is necessary to obtain some indication of the total ChE activity in intact preparations. For any particular preparation, however, it would be necessary to ascertain to what degree the particular venom used increases permeability.

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