Demonstration of Increased Permeability as a Factor in the Effect of Acetylcholine on the Electrical Activity of Venom-Treated Axons

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ABSTRACT D-Tubocurarine (curare) and acetylcholine (ACh) had been found to block electrical activity after treatment of squid giant axons with cottonmouth moccasin venom at a concentration which had no effect on conduction. It has now been demonstrated that this effect is attributable to reduction of permeability barriers. The penetration of externally applied C^{14}-labeled dimethylcurare, ACh, choline, and trimethylamine into the axoplasm of the squid giant axon was determined in axons treated with either cottonmouth, rattlesnake, or bee venom, and in untreated control axons. The lipid-soluble tertiary nitrogen compound trimethylamine readily penetrated into the axoplasm of untreated axons. In contrast, after exposure of the axons to the lipid-insoluble quaternary nitrogen compounds for 1 hour their presence in the axoplasm was hardly detectable (less than 1 per cent). However, following 15 μg/ml cottonmouth venom 1 to 5 per cent of their external concentration is found within the axoplasm while following 50 μg/ml venom 10 to 50 per cent enters. The penetration of dimethylcurare is also increased by 10 μg/ml bee venom but not by 1 μg/ml bee venom nor 1000 μg/ml rattlesnake venom. The experiments show that when ACh and curare, following venom treatment, affect electrical activity, they also penetrate into the axon. Treatments which do not increase penetration are also ineffective in rendering the compounds active.

INTRODUCTION

The inability of acetylcholine (ACh), d-tubocurarine (curare), and other lipid-insoluble quaternary nitrogen compounds to affect electrical activity in axons such as those of the frog sciatic nerve or squid stellar nerve has been thought to be incompatible with the view of an essential role of ACh in axonal conduction. This, however, has been explained by Nachmansohn (1) as being due to structural barriers which prevent lipid-insoluble compounds from reaching the conducting membrane. He and his associates have shown

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that ACh labeled with N^{15}, or neostigmine, fails to penetrate into the axoplasm of the squid giant axon whereas the lipid-soluble tertiary nitrogen compounds, physostigmine and trimethylamine, enter readily (2, 3).

Recently, however, evidence has accumulated that ACh and curare may affect electrical activity if either special preparations are used where barriers, if present at all, are relatively weak or if the axons are treated with agents presumably reducing the permeability barriers. Dettbarn (4) has demonstrated that at Ranvier nodes of a single frog sciatic nerve fiber curare rapidly and reversibly blocked electrical activity. Armett and Ritchie found that the desheathed vagus of the rabbit is sensitive to ACh (5, 6). Dettbarn and Davis (7) found a direct action of ACh on the axons of the walking leg of the lobster. Walsh and Deal reported that after exposure of the desheathed frog sciatic nerve to cetyltrimethylammonium bromide, curare, ACh, and other compounds reversibly blocked conduction (8).

It has recently been shown that if the squid giant axon is pretreated with *Agkistrodon p. piscivorus* (cottonmouth moccasin) venom, ACh, curare, and dimethylcurare reversibly block conduction (9). It was pertinent therefore to determine directly whether the treatment with moccasin venom does in fact allow these compounds to penetrate into the axon.

MATERIALS AND METHODS

Giant axons of the squid (*Loligo pealii*) were dissected free of the fin nerve and most but not all the small nerve fibers. About 70 mm lengths of axons were ligated at both ends and placed in a nerve chamber used for external recording of electrical activity (9). Some axons were exposed to a solution of 15 μg/ml of moccasin venom for 30 minutes which produced little or no effect upon electrical activity, while other axons were placed in a solution of 50 μg/ml until electrical activity was blocked (20 to 40 minutes). In addition some axons were exposed for 30 minutes to 1000 μg/ml *Crotalus adamanteus* (eastern diamondback rattlesnake) venom or 10 μg/ml of bee venom. Following 10 minutes' rinsing in normal sea water all axons were placed in 25 ml of sea water containing a known amount of one of four radioactive compounds diluted with non-radioactive carrier to give the final concentration of the compounds noted in the Results. The axons were incubated in this solution for 1 hour, during and after which electrical activity was checked. The axon was then removed from the chamber, passed through three sea water washings (1 minute), blotted on filter paper, and one end cut open. This end for a length of about 10 mm had been suspended out of contact with the radioactive solution in the nerve chamber in order to decrease the possibility of contamination during the process of cutting the axon open. This might make the absolute amounts of penetration appear slightly lower than they actually were but would not change the relative amounts. The axon was placed on a microscope slide and held in a vertical position with about 5 mm of the cut end of the axon hanging free below the slide. A tygon-coated roller was used to extrude the axoplasm onto a weighed microscope cover glass, being careful that only the extruded axo-

plasm, and not the axon, touched the cover glass. The room temperature during these experiments was about 20°C. The fresh sample was weighed, and assayed for radioactivity within the hour. C^{14} was assayed in the form of CO_2 gas after wet oxidation by the technique of Van Slyke and coworkers (10). In the experiments with trimethylamine it was necessary to apply a drop of dilute mineral acid to the sample of axoplasm immediately after weighing in order to prevent loss of trimethylamine as a gas. All disintegration values recorded in the Results are corrected for background radiation.

TABLE I

PENETRATION OF ACETYLCHOLINE (ACh) INTO AXOPLASM OF SQUID
AXON FOLLOWING COTTONMOUTH VENOM TREATMENT

Each axon exposed to a radioactivity of 8.2×10^5 disintegrations/min./ml
of solution and 4.4×10^{-2} M ACh bromide (plus 2.4×10^{-4} M non-radioactive
physostigmine salicylate) for 1 hour. If the axonal membrane were to offer no
barrier to free diffusion, a concentration of 4.4×10^{-8} moles ACh would be
expected per milligram of fresh axoplasm.

Cottonmouth moccasin venom	Axoplasm	Disintegrations/min./sample		Penetration
		Expected if no barrier*	Found	
µg/ml	mg			per cent
0	7.10	5822*	17	0.3
0	4.94	4051	31	0.8
0	3.55	2911	20	0.7
15	8.72	7150	167	2.3
15	6.49	5322	77	1.5
15	5.20	4264	143	3.4
15	5.94	4871	53	1.1
50	8.82	7232	4330	59.9
50	7.87	6453	1011	15.7
50	4.25	3485	405	11.6

* Assuming for the calculation that 1 mg of axoplasm contains 1 µl of solution into which ACh can diffuse.
Example $(8.2 \times 10^5) \times (7.1 \times 10^{-3}) = 5822$.

Dimethyl-C^{14}-d-tubocurarine diiodide, choline (methyl-C^{14}) chloride, C^{14}-trimethylamine hydrochloride, and acetylcholine (N-methyl-C^{14}) chloride were obtained from New England Nuclear Corporation. Lyophilized venoms of *Agkistrodon p. piscivorus* (lot 6/29/61) and *Crotalus adamanteus* (lot 2/2/61) were purchased from Ross Allen Reptile Institute at Silver Springs, Florida. Bee venom was purchased from Nutritional Biochemicals Company. Crystalline non-radioactive dimethyl-d-tubocurarine iodide was purchased from Eli Lilly and Company.

RESULTS

As shown in Table I moccasin venom, in the concentrations previously used to render ACh and curare active in the electrical experiments (9), does

increase the rate of penetration of ACh into the axoplasm. An even greater increase in the penetration of ACh is obtained with 50 μg/ml venom, a concentration previously shown to block conduction (9). ACh had no effect on electrical activity of the control axons; following 15 μg/ml venom treatment it blocked conduction in two of the four axons. Physostigmine was added in a concentration which has no effect on electrical activity (9, 11), but which further reduces the already low rate of ACh hydrolysis by squid axon (12).

The increased penetration of choline into the axoplasm following moccasin venom is shown in Table II. Choline is of special interest because of its relation to ACh and because it is rather inert, causing in 1 hour only about a

TABLE II

PENETRATION OF CHOLINE INTO AXOPLASM OF SQUID AXON
FOLLOWING COTTONMOUTH VENOM TREATMENT

Each axon exposed to a radioactivity of 3.72 × 10⁶ disintegrations/min./ml of solution and 5.4 × 10⁻² M choline bromide for 1 hour. If the axonal membrane were to offer no barrier to free diffusion a concentration of 5.4 × 10⁻⁸ moles choline would be expected per milligram of fresh axoplasm.

| Cottonmouth moccasin venom | Axoplasm | Disintegrations/min./sample | | Penetration |
		Expected if no barrier	Found	
$\mu g/ml$	mg			$per\ cent$
0	5.12	1905	3	0.2
0	7.02	2611	14	0.5
15	4.46	1659	236	14.2
15	4.21	1566	60	3.8
50	3.68	1369	660	48.2
50	5.21	1938	621	32.1

30 per cent decrease in spike height when applied in a concentration of 0.054 M after 15 μg/ml venom. See also reference 9.

Both curare and dimethylcurare block conduction of squid axon following moccasin venom treatment (9); however, only the latter was available to us in radioactive form. The results obtained are shown in Table III. Dimethylcurare caused about a 75 per cent decrease in spike height in the two axons exposed to 15 μg/ml venom. Bee venom at 10 μg/ml blocked electrical activity in less than 15 minutes, while 1 μg/ml bee venom and 1000 μg/ml rattlesnake venom had no effect in 30 minutes. Dimethylcurare had no effect on the propagated spike potential following rattlesnake venom or 1 μg/ml bee venom. These observations are in agreement with previous results (9, 13).

The penetration of trimethylamine was retested in order to compare the present results with those obtained previously with slightly modified experi-

TABLE III

PENETRATION OF DIMETHYL-D-TUBOCURARINE INTO AXOPLASM
OF SQUID AXON FOLLOWING VENOM TREATMENT

Each axon exposed to a radioactivity of 5.2 × 10⁵ disintegrations/min./ml of
solution and 1.1 × 10⁻³ M dimethyl-d-tubocurarine diiodide for 1 hour. If the
axonal membrane were to offer no barrier to free diffusion a concentration of
1.1 × 10⁻⁹ moles dimethyl-d-tubocurarine would be expected per milligram
of fresh axoplasm.

| Venom | Concentration | Axoplasm | Disintegrations/min./sample | | Penetration |
			Expected if no barrier	Found	
	µg/ml	*mg*			*per cent*
None	0	4.01	2085	4	0.2
None	0	5.05	2626	15	0.6
None	0	7.07	3676	7	0.2
Cottonmouth moccasin	15	5.48	2850	133	4.7
Cottonmouth moccasin	15	5.31	2761	84	3.1
Cottonmouth moccasin	50	4.87	2532	364	14.4
Cottonmouth moccasin	50	5.28	2746	90	3.3
Cottonmouth moccasin	50	5.72	2974	276	9.3
Cottonmouth moccasin	50	9.38	4878	2745	56.5
Rattlesnake	1000	5.67	2948	26	0.9
Rattlesnake	1000	3.55	1846	8	0.4
Bee	1	3.28	1706	2	0.1
Bee	1	3.16	1643	0	0.0
Bee	10	3.91	2033	494	24.3
Bee	10	3.01	1565	449	28.8

mental techniques. The earlier report (3) that trimethylamine readily pene-
trates into the axoplasm of the squid axon, in contrast to ACh, was fully
confirmed (Table IV).

TABLE IV

PENETRATION OF TRIMETHYLAMINE
INTO AXOPLASM OF SQUID AXON

Each axon exposed to a radioactivity of 1 × 10⁶ disintegrations/min./ml of
solution and 1.15 × 10⁻² M trimethylamine hydrochloride for 1 hour. If the
axonal membrane were to offer no barrier to free diffusion a concentration
of 1.15 × 10⁻⁸ moles trimethylamine would be expected per milligram of
fresh axoplasm.

| Cottonmouth moccasin venom | Axoplasm | Disintegrations/min./sample | | Penetration |
		Expected if no barrier	Found	
µg/ml	*mg*			*per cent*
0	1.20	1200	1359	113.2
0	1.47	1470	1712	116.5
50	1.49	1490	1422	95.4
50	0.76	760	876	115.3

DISCUSSION

The demonstration that ACh and curare affect electrical activity after exposure of squid giant axons to moccasin venom, whereas these compounds are completely inactive without treatment, has been interpreted as being due to a reduction of the permeability barrier preventing lipid-insoluble compounds from entering the axon and reaching the conducting membrane (9). In view of the crucial question involved, viz. whether ACh and curare act on the axonal membrane, this interpretation has been submitted to a direct test; i.e., whether the venom which has been shown to allow ACh and curare to affect electrical activity also allows them to penetrate.

The data presented in this paper have clearly established that ACh and curare do penetrate after treatment with the low concentration of cottonmouth moccasin venom which renders these compounds active. The phospholipase A component of the venom is probably responsible for these effects (9, 14). In contrast, after exposure to rattlesnake venom, even in high concentration, curare neither penetrates nor does it affect electrical activity. Bee venom in concentrations which do not block electrical activity does not render curare active (13), nor does it increase curare penetration (Table III). Thus the results provide direct evidence that compounds expected to interact with the ACh system will affect conduction if they are able to penetrate to the active membrane. In support of this idea it had previously been found that lipid-soluble tertiary nitrogen compounds do penetrate into the axoplasm of the squid axon (2, 3), and those which would be expected to interact with the ACh system block conduction (2, 11). The effective concentrations are similar to those required to block transmission in the electroplaque synapse (15, 16). The effects of ACh appear rather specific since choline is relatively inactive on the squid axon after venom pretreatment (9), even though its penetration is increased by moccasin venom (Table II). This is in agreement with the much greater sensitivity of synaptic receptors to ACh than to choline.

The increased permeability produced by the venoms agrees with suggestions made many years ago, even before any evidence was available, that many of the effects of venoms are due to increased permeability of biological membranes. More recently it has been observed that moccasin venom increases the passage of perfusion fluid through frog atria (17), the penetration of procaine into frog sciatic nerves (18), and hemolyzes red blood cells (19). Some references to the earlier venom literature are noted in a recent book (20).

In control axons the penetration of ACh, choline, and dimethylcurare is less than 1 per cent of that expected if no barrier were present. Even these low values, however, may not represent actual penetration but may be due to contamination during the process of extrusion, or the presence of trace

amounts of C^{14}-tertiary nitrogen containing compounds as trace impurities in the radioactive samples of the quaternary compounds. No effort was made to tie off or dissect very carefully the small nerve branches of the squid axon. Any penetration through the cut ends is minimal however, as evidenced by the controls.

The receptor areas of the membrane are external to the axoplasm. Although only 70 to 80 A thick (21, 22), the membrane is complex and the exact location of the ACh system is not known. It is impossible to know what the concentration of ACh and dimethylcurare may be in the membrane compared to the 1 to 5 per cent found in the axoplasm after 15 μg/ml cottonmouth venom. Even the meaning of the term concentration in such a membrane is uncertain. Considering, however, the wide variety of compounds to which nerve axons may be exposed in the body, the receptor areas of the membrane may be especially protected against the action of external compounds. It appears idle therefore to speculate about the penetration rate in terms of concentration at the active site. It is not satisfactory to measure the concentration of these compounds in the whole nerve as a test for this hypothesis since one would expect non-specific binding of charged molecules such as ACh and curare with many macromolecules external to the active neuronal membrane.

Tasaki and Spyropoulos (23) have determined the radioactivity of axons (not axoplasm) following exposure to C^{14}-choline in the external medium and observed a time-dependent increase in radioactivity of the whole axon which in 1 hour reached about 10 per cent of that in the external medium. This procedure, however, does not distinguish between a real penetration of choline into the membrane and a non-selective binding to sites, *e.g.* nucleic acid, chondroitin sulfate, etc., external to the active neuronal membrane, with which any charged molecule might be expected to interact. The same authors also injected C^{14}-choline into the axoplasm and detected about 10 per cent of it in the external media in 10 minutes. There is thus a considerable difference between our results and those of Tasaki and Spyropoulos (23). It is possible that the permeability barriers are not as impervious in the direction from axoplasm to external medium as in the reverse direction. The authors used moreover very finely dissected axons in contrast to the relatively crudely dissected preparations we employed and the permeability barriers may have been slightly different. An additional possibility is that some choline was enzymatically demethylated in the axoplasm to its lipid-soluble analogue, which then penetrated through the membrane. When choline is applied to the external solution, such enzymatic action would not be expected to occur.

The data presented lend new support to the view of the importance of the

ACh system in axonal conduction. The results must, of course, be considered in connection with other evidence as for instance the presence of the entire ACh system in conducting tissues (1) and the block of electrical activity by powerful and specific inhibitors of ACh-esterase (physostigmine, organophosphorus compounds) (1), or of the ACh receptor (local anesthetics) (24). One of the main objections to the proposed role of ACh was the failure of direct action; the present results have removed this difficulty by the evidence that the lipid-insoluble compounds do have the postulated action, provided they are able to penetrate.

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