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PENETRATION OF ACETYLCHOLINE INTO SQUID GIANT AXONS*

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Abstract—Acetylcholine (ACh) neither blocks conduction of nor penetrates into the axoplasm of crudely or finely dissected squid giant axons. *d*-Tubocurarine (curare) is also unable to affect conduction even in finely dissected axonal preparations. The potency of physostigmine is about equal in the two preparations. These results indicate that adhering small nerve fibers and connective tissue may not be a strong permeability barrier. Cetyltrimethylammonium chloride (CTA) is more potent on finely dissected than on crudely dissected preparations, whereas cottonmouth moccasin venom is much more potent on the crudely dissected preparation. ACh and curare markedly depress conduction in finely dissected axons pretreated with CTA but not in those pretreated with venom. CTA apparently disrupts permeability barriers directly, whereas the venom acts indirectly, probably by forming lysophosphatides in the adhering small nerve fibers and connective tissue. Removal of calcium and magnesium from the seawater increases the penetration of ACh through the axonal membrane, which may partially explain the known antagonism between curare and these ions on certain biological preparations.

THE electrical activity of the squid giant axon is unaffected by the external application of even very high concentrations of acetylcholine (ACh),*d*-tubocurarine (curare) and other lipid-insoluble quaternary nitrogen compounds;[1-4] it is, however, readily affected by lipid-soluble tertiary nitrogen compounds such as physostigmine[1, 5] and atropine,[1] which are thought to interact at junctions with ACh esterase or the ACh receptor respectively. This apparent discrepancy with the unified theory of the role of ACh in conduction[3,4] was explained by the finding that ACh and related quaternary nitrogen compounds cannot penetrate through the squid membrane, whereas tertiary nitrogen compounds readily penetrate.[5-7] Additional evidence that permeability barriers are responsible for the inability of ACh and curare to affect conduction was obtained by reducing the barriers either with certain snake venoms or the detergent cetyltrimethylammonium chloride (CTA) and then demonstrating block of conduction with ACh, curare, and other compounds.[1, 2, 7-9]

Most of these studies have been carried out on squid stellar nerve with many small nerve fibers attached to the giant axon. It has been reported that ACh readily penetrates into the axoplasm of finely dissected squid giant axons (Mitchell *et al.*);[10]

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electrical activity was not recorded in these experiments. Since these results are in contrast to the findings with more crudely dissected axons, it was of interest to determine the effects of Ach on electrical activity of, and the penetration of ACh into, finely dissected axons. One of the reasons for having used relatively crudely dissected axons was the inertness of cottonmouth venom on electrical activity of finely dissected axons. In contrast, when crudely dissected axons were used, the venom rendered them sensitive to ACh and curare[2] and produced by itself, in higher concentrations, irreversible block. In this paper these problems have been further investigated and experiments are reported in which cottonmouth venom was applied to the finely dissected squid giant axon.

MATERIALS AND METHODS

The procedure used for crude dissection of the stellar nerve involves removing the majority but not all of the small fibers adhering to the giant axon.[1] In the finely dissected preparations used in this study special care was taken to remove all the small nerve fibers without damaging the giant axon membrane. External recordings of electrical activity were obtained by placing a nerve on five Ag–AgCl electrodes in a 25-ml chamber—two recording, two stimulating, and one ground electrode.[1, 2] Every 5 or 10 min the seawater was removed and the axonal action potentials were recorded by stimulating a few times with pulses of 0·1 msec duration and of controlled intensity. At the minimal voltage required to stimulate the giant axon, the small nerve fibers did not respond. The electrical activity of the axons was monitored for 30 min after dissection and prior to beginning any experiment, in order to make sure they had not been injured during the dissection.

Solutions of the various materials were freshly prepared with filtered natural seawater buffered at pH 7·5–8·0 with 1 mM Tris. As previously found, this amount of Tris had no effect on the spike height.[1] When artificial seawater was used it contained the following composition in millimoles per liter: NaCl, 423; KCl, 9·00; $CaCl_2 \cdot 2H_2O$, 9·27; $MgCl_2 \cdot 6H_2O$, 22·94; $MgSO_4 \cdot 7H_2O$, 25·50; $NaHCO_3$, 2·15. In some experiments the calcium and magnesium were either reduced or eliminated; temperature was 17° to 21°.

In the axons pretreated with venom or detergent the agents were applied for 30 min, during which time electrical activity was monitored. The axons were then placed in normal seawater for at least 10 min, after which the compound under investigation was applied for 30 or 60 min or until block of electrical activity occurred. Reversibility was checked by adding a fresh sample of seawater for 30 or more min.

In the ^{14}C-penetration studies axons were exposed in a 25-ml chamber to N-methyl-^{14}C-labeled ACh of known specific gravity. Details of the techniques used were previously described.[7, 9] In those experiments little or no ^{14}C was found in the axoplasm of the control axons, indicating that the ^{14}C-methyl groups were not split off the ACh nor entering the one carbon metabolism of the axon. In the venom-treated axons, significant radioactivity was found in the axoplasm and it is possible that, after penetration of the ^{14}C-ACh, enzymes in the axoplasm or membrane split off the methyl groups. This would not, however, interfere with our measurements of the penetration of ACh, since our control experiments indicated that if any such action occurred, it happened only subsequent to the penetration of ACh. Physostigmine

($2 \cdot 4 \times 10\text{-}^4$ M), which itself had no effect on electrical activity, was added to decrease the rate of hydrolysis by ACh esterase.

After an interval of time in the ACh solution, usually 60 min, the axons were removed, passed through three seawater washings (1 min), blotted on filter paper, and one of the ligated ends 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. 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. The fresh sample was weighed and assayed for radioactivity within the hour. ^{14}C was measured as CO_2 gas after wet oxidation by the technique of Van Slyke and co-workers.[11] The very low levels of ^{14}C-ACh penetration in control axons[7, 9] indicate that at most only slight contamination may have occurred during the process of extrusion of the axoplasm.

The radioactivity of the solution to which the axons were finally exposed was $3 \cdot 0 \times 10^5$ disintegration/min/ml; the ACh concentration varied, as listed in Table 2, from 9×10^{-2} to 9×10^{-5} M, this being accomplished by dilution of the purchased ^{14}C-ACh with known amounts of nonradioactive ACh. Usually about 3 to 5 mg of axoplasm was extruded (range 1·5 to 10·5 mg). The disintegrations per minute expected in the axoplasm if there were no barrier to the penetration of ^{14}C-ACh ranged from about 400 to 4,000 dependent on the final specific activity and weights of axoplasm extruded, and assumed for the calculation that 1 mg of axoplasm is equivalent to 1 μliter of solution into which ACh can diffuse. The actual disintegrations/min/sample above background found in the axoplasm were used for calculating the per cent penetration; that is, the percentage of the external concentration prevailing within the axoplasm. In some experiments following extrusion of the axoplasm the envelope (i.e. membrane. associated cell-wall material, and connective tissue) was assayed for radioactivity. The average weight of the envelope was about 0·4 mg (range, 0·2 to 1·0 mg), giving an expected radioactivity above background of about 90 to 400 disintegrations/min.

All results are recorded in the tables as means $\pm$ S.E. (standard error or standard deviation of the mean).

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

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

Acetylcholine (N-methyl-C^{14}) chloride was obtained from New England Nuclear Corp. Lyophilized cottonmouth moccasin venom (*Agkistrodon p. piscivorous*, lot 7-9-63) was purchased from Ross Allen Reptile Institute, Silver Springs, Fla.

RESULTS

The effects of several compounds on the action potential of finely and crudely dissected squid axons are shown in Table 1. Curare and ACh were inactive on both preparations, while the potency of physostigmine was similar in both. As reported previously,[2] cottonmouth venom is practically inert on finely dissected squid axons.

In contrast CTA is considerably more potent on finely dissected axons than on those crudely dissected.

In addition to the results given in Table 1, the effects of $4{\cdot}5\times10^{-2}$ M ACh and $1{\cdot}4\times10^{-3}$ M curare were checked on the electrical activity of finely dissected axons pretreated for 30 min with 250 μg venom/ml (six experiments). They each caused only

TABLE 1. EFFECTS OF VARIOUS AGENTS ON THE ACTION POTENTIAL (A.P.) OF FINELY DISSECTED (F.D.) AND RELATIVELY CRUDELY DISSECTED (C.D.) SQUID GIANT AXON

Agent	Conc. (M)	No. of expts.		Exposure (min)	Decrease of A.P.		Reversal
		C.D.	F.D.		C.D. %	F.D.	%
Curare	$1{\cdot}4\times10^{-3}$	3	3	30±0	0±0	13±6	
Acetylcholine	$4{\cdot}5\times10^{-2}$	4	4	30±0	0±0	0±0	
Cottonmouth moccasin venom	100 µg/ml	4		17±3	100±0		0±0
	500 µg/ml		4	60±0		9±4	
CTA	$2{\cdot}4\times10^{-4}$	3		20±5	95±5		0±0
	$9{\cdot}6\times10^{-5}$	3		30±0	5±2		
	$9{\cdot}6\times10^{-5}$		3	18±8		100±0	0±0
	$2{\cdot}4\times10^{-5}$		3	30±0		30±5	0±0
Physostigmine	$4{\cdot}8\times10^{-3}$	2	2	30±0	30±0	35±3	100±0
	$1{\cdot}2\times10^{-2}$	3		18±4	75±0		90±5
	$1{\cdot}2\times10^{-2}$		2	25±0		100±0	80±10

All results are given as mean ± standard deviation of the mean (standard error). CTA=Cetyltrimethylammonium chloride.

a small decrease (less than 20%) of the action potential in 30 min, which contrasts markedly with their potent effects on crudely dissected axons pretreated with 15 or 25 μg venom/ml.[2, 8] It was also observed, in three experiments following pretreatment of the finely dissected axon with $2{\cdot}4\times10^{-5}$ M CTA for about 20 min, that curare ($1{\cdot}4\times10^{-3}$ M) caused about a 70% decrease of the action potential in 20 min. In principle, this is similar to previous findings with crudely dissected axons except that the concentration of CTA required in these finely dissected axons was lower.

The penetration of ACh into the axoplasm of the squid giant axon under various conditions is shown in Table 2. The results confirm the previous findings[7, 9] that cottonmouth venom markedly increases the penetration of ACh into crudely dissected axons. In contrast, even a relatively high concentration of venom has no effect on ACh penetration into finely dissected axons. The penetration of ACh into finely dissected axons is as low as that observed in the crudely dissected preparations which were not pretreated with venom. The per cent penetration of ACh into crudely dissected and venom-pretreated axons was similar over a thousandfold range of concentration. In axons pretreated with 100 μg venom/ml the penetration of ACh appears to increase with time, while without venom treatment the apparent penetration of ACh in 5 min appears to be as great as that found in 60 min (Table 2 and Refs. 7, 9).

Removing calcium and magnesium from artificial seawater seems to increase the penetration of ACh ($P<0{\cdot}02$). A marked alteration of pH of seawater does not appear to change the penetration; in any event, in the few experiments performed, the differences between the penetration at pH 5 or 10 and pH 7·5 to 8·0 (S.W.

condition) are not significantly different ($P>0\cdot10$). Seawater alone at pH 5 or 10 did not alter the height of the conducted action potential in 1 hr. In seven other experiments it was found that $4\cdot5\times10^{-3}$ M ACh in pH 5 or pH 10 seawater caused only about a 10% decrease in the action potential in 25 min. It is very difficult to determine effects on electrical activity by ACh in seawater free of calcium and magnesium since this modified seawater itself will markedly depress the height of the conducted action potential, as noted in Table 2, although this effect is often reversible.

TABLE 2. PENETRATION OF ACh INTO AXOPLASM OF SQUID GIANT AXON

Venom (μg/ml)	Condition	ACh (M)	No. of expts.	Decrease in A.P. by ACh (%)	Penetration (%)
25		$9\cdot0\times10^{-5}$	2	50±10	6·2±1·7
25		$4\cdot5\times10^{-3}$	3	87±4	5·5±1·3
25	*	$4\cdot5\times10^{-3}$	3	100±0	13·2±6·1
25		$9\cdot0\times10^{-2}$	2	100±0	7·5±0
0		$4\cdot5\times10^{-3}$	2	0±0	0·5±0·3
0	5-min exp.	$4\cdot5\times10^{-3}$	3	11±8	1·9±1·2
0	pH 5	$4\cdot5\times10^{-3}$	2	22±6	5·4±2·9
0	pH 10	$4\cdot5\times10^{-3}$	2	31±15	3·2±0·5
0	S.W.	$4\cdot5\times10^{-3}$	2	0±0	2·1±0·2
0	S.W., no Ca, Mg	$4\cdot5\times10^{-3}$	7	†	4·7±0·9
100	5-min exp.	$4\cdot5\times10^{-3}$	2	‡	14·5±6·9
100	20-min exp.	$4\cdot5\times10^{-3}$	2	‡	33·8±6·2
0	F.D.	$4\cdot5\times10^{-3}$	4	10±3	1·1±0·4
250	F.D.	$4\cdot5\times10^{-3}$	4	22±7	1·6±0·4

Each axon exposed to C^{14}-ACh ($3\cdot0\times10^{5}$ disintegrations/min/ml of solution) and nonradioactive AChBr plus $2\cdot4\times10^{-4}$ M physostigmine salicylate. Some axons were pretreated with cottonmouth moccasin venom for 30 min in concentrations shown above; 100 μg venom/ml blocked conduction while the other concentrations noted above had no effect on the conducted action potential (A.P.). Except as shown above under "condition" column, all conditions were as follows: relatively crudely dissected squid giant axons were used; axons were exposed (exp.) to ACh for 60 min; pH=7·5–8·0; filtered natural seawater used. All results are given as mean ± standard deviations of the mean (standard error). F.D.=finely dissected squid axon. S.W.=artificial seawater. Sucrose was nonradioactive. The results of the two experiments using $4\cdot5\times10^{-3}$ M ACh under standard conditions in axons which were not pre-treated with venom are taken from earlier data[9].

*Sucrose, 9×10^{-3} M.

†Seawater without Ca^{2+} and Mg^{2+} caused a 74±6% decrease in A.P. and some spontaneous firing.

‡Venom by itself blocked conduction.

In five experiments in addition to those listed in Table 2, seawater free of calcium and magnesium caused about a 70% reversible decrease of the action potential in 10–20 min in crudely dissected axons. Few or no spontaneous firings were observed. Spontaneous firings at a rate of about 100/sec consistently occurred in seawater containing 4·6 or 2·3 mM Ca and 24 or 12 mM Mg instead of 9·3 mM Ca and 48·4 mM Mg, while there were respectively 0% and 20% decreases in the action potential in 15 min (eight experiments). After pretreatment with 25 μg cottonmouth venom/ml, decrease of Ca^{2+} or Mg^{2+} had a much more pronounced effect: seawater with 0% or 25% of the normal amounts of calcium and magnesium blocked conduction within 3 min irreversibly (five experiments); with 50% of the normal amounts there was about a 60% decrease in the action potential in 10–15 min. No spontaneous firings were observed in any of these experiments.

The effects of $4{\cdot}5\times10^{-3}$ M ACh and $1{\cdot}4\times10^{-3}$ M curare, in seawater free of calcium and magnesium or containing 25% of the normal concentrations, was tested in crudely dissected axons which had not been venom pretreated. They appeared to cause no greater decreases in the conducted action potential than the modified seawater itself (eight experiments).

The uptake of ^{14}C-ACh by the envelope of the finely dissected squid axon after extrusion of the axoplasm was $47\pm11\%$ of that in the external solution (three experiments). It was assumed as for axoplasm that the weight of the envelope in milligrams is equal to the same volume of bathing medium in microlitres.

DISCUSSION

The data presented are in contrast to those reported by Mitchell *et al.*[10] that ACh rapidly enters into the axoplasm of finely dissected axons: the penetration of ACh was about equally low in both finely and crudely dissected axons. Even this low level of apparent penetration may not represent actual penetration but may be due to contamination occurring during extrusion of axoplasm[7]. This suggestion is supported by our finding that the penetration of ACh in venom-treated axons appears to increase with increasing time of ACh incubation, whereas in axons not pretreated with venom, ACh penetration after 5 min incubation is as great as after 60 min. The amount of contamination occurring during extrusion of axoplasm would be expected to remain approximately constant regardless of whether the ACh incubation time was 5 or 60 min. In the studies of Mitchell *et al.*[10] the electrical activity of the axons was not checked. This makes the meaning of their experiments questionable.

Since ACh did not penetrate even in the finely dissected preparation it is not surprising that it did not affect conduction. Curare was likewise inactive on the finely dissected preparation, while the potency of physostigmine was about equal in crudely and finely dissected axons. These results indicate that the adhering small nerve fibers and associated connective tissue do not constitute a strong permeability barrier for the giant axon. The major permeability barrier is apparently the surrounding Schwann cell. Recent, as yet unpublished, electron micrographs taken by Dr. David Robertson of Harvard University indicate that cottonmouth venom disrupts the Schwann cell with little or no effect on the axolemma, suggesting therefore that the Schwann cell is the most effective permeability barrier.

Previous studies indicate that the active component of cottonmouth venom is phospholipase A.[2, 12] It was also observed, as has now been confirmed, that cottonmouth venom was relatively inactive in finely dissected axons, in contrast to its potency on crudely dissected preparations. These findings led to the suggestion that the venoms are acting through the formation in or near the membrane of a secondary product, probably a lysophosphatide which in turn affects electrical activity and permeability by disrupting barriers near the giant axon membrane. In the closely dissected axon there would be less substrate on which phospholipase A could act and therefore fewer lysophosphatides would be formed. A similar type of explanation is used to explain the well-known observation that venom or phospholipase A will not hemolyze washed red blood cells, whereas the addition of lecithin or serum to the red blood cells rapidly leads to hemolysis by phospholipase A. This explanation also accounts for the finding (see Results) that ACh neither affects electrical activity nor

penetrates into the axoplasm of finely dissected and venom-treated squid giant axons. This explanation is also favored by the finding that the detergent CTA, which does act directly, was even more potent on the finely dissected axon than on the crudely dissected preparation, in contrast to the findings with cottonmouth venom. The effects of CTA on the electrical properties of the finely dissected squid axon membrane have been analyzed, with concentrations of CTA similar to those which we found effective.[13]

Physostigmine, neostigmine, and choline markedly decrease the penetration of ACh into the axoplasm of the venom-treated squid axon and decrease the effects of ACh on conduction.[9] It was suggested that they compete for sites of penetration. It was of interest to investigate whether there was a degree of specificity about this competition phenomenon. The penetration of ACh was therefore tested in the presence of sucrose, a pharmacologically indifferent compound. As noted in Table 2, sucrose did not decrease penetration but may have actually increased it, although the difference is not significant in the few experiments performed. This result would therefore indicate that charged molecules may more effectively compete with ACh for penetration sites than uncharged molecules. Not all charged molecules will, however, produce similar effects; curare, for instance, increased rather than decreased the penetration of ACh[9]. This is not surprising in view of the great differences of the properties and structures of charged molecules.

Calcium antagonizes the action of curare at junctions,[14] and decreasing the calcium and magnesium in seawater allows curare to block conduction in nerves from the walking leg of lobsters, whereas in normal seawater, curare is without effect on this preparation[15]. There may be a direct competition for receptor sites but in addition our results indicate that Ca and Mg interfere with the penetration of ACh, since the penetration appears to be greater in seawater free of Ca and Mg. A similar interference in the penetration of curare could be at least a partial cause for the findings on junctions and lobster nerves noted above. In our studies with seawater free of Ca and Mg it was difficult to assess the effects of ACh and curare on electrical activity because of the direct effects of this modified seawater on conduction. Cottonmouth venom apparently increases the permeability of the membrane to calcium, magnesium, and possibly other ions as evidenced by the markedly enhanced effects on venom-treated axons of seawater with reduced concentrations of Ca and Mg. This may be due to the greater removal of Ca and Mg from the area of the axonal membrane when the venom-treated axon is bathed in seawater containing reduced concentrations of Ca and Mg.

A considerable amount of ^{14}C–ACh was found in the envelope of the squid axon. Since there are, no doubt, many negatively charged sites in the connective tissue and Schwann cell surrounding the axolemma as well as in the axolemma itself, these results probably indicate nonspecific binding or adsorption rather than binding on specific receptor sites. The meaning of this observation is doubtful, but it shows the justification for measuring in these and previous studies ^{14}C in the axoplasm rather than in the whole axon.

The results presented in this paper provide further support for the idea that quaternary nitrogen compounds which would be expected to interact with the ACh system will affect conduction provided they are able to penetrate. The inability of ACh and related compounds to affect conduction in certain axons can therefore no longer be used as an argument against the proposed role of ACh in conduction[3, 4].

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