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PENETRATION OF NEOSTIGMINE, PHYSOSTIGMINE, AND PARAOXON INTO THE SQUID GIANT AXON*

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Abstract—The penetration of the cholinesterase inhibitors neostigmine, physostigmine, and paraoxon into the axoplasm of the squid giant axon has been determined by measuring cholinesterase inhibition with the magnetic diver technique. Neostigmine (5×10^{-2} M) neither penetrated significantly into control axons nor into axons pretreated with 25 μg cottonmouth moccasin venom/ml, and neostigmine did not affect conduction. Significant penetration of neostigmine (0·5–1·0 per cent) was observed after pretreatment of axons with a concentration of venom (100 μg/ml) which itself blocked conduction. The approximate levels in the axoplasm of paraoxon (10^{-2} and 10^{-3} M) were 5 per cent of those in the external media, whereas physostigmine ($2{\cdot}5 \times 10^{-3}$ and 10^{-5} M) attained levels about 10 times as great. Only the higher concentrations of the two inhibitors affected conduction. The apparent discrepancy between penetrability and blocking potency is discussed.

ACETYLCHOLINE (ACh), *d*-tubocurarine (curare), neostigmine, and other quaternary nitrogen compounds do not affect electrical activity of most axonal preparations, e.g. frog sciatic and squid giant axons. This has been thought to be incompatible with the view of the essential and specific role of ACh in the permeability changes of excitable membranes during electrical activity, as postulated by Nachmansohn.[1] He and his associates have, however, offered evidence for the existence of structural permeability barriers preventing these lipid-insoluble compounds from penetrating into the axoplasm through the cell wall surrounding the squid axon membrane.[2–4] Obviously, if compounds are unable to penetrate it appears reasonable to assume that their inability to affect the events in the plasma membrane is due to their inability to reach it. After exposure of frog sciatic nerves to a detergent, Walsh and Deal observed reversible block of conduction by ACh, curare, neostigmine, and other quaternary nitrogen derivatives.[5] Studies over the past several years, carried out with the squid giant axon, have shown after the application of cottonmouth moccasin venom, in a concentration which has no effect on conduction, that ACh, curare, decamethonium, and other compounds which are normally inactive will reversibly block conduction.[6–8] This effect has been correlated with the increased penetration of ^{14}C labeled ACh and dimethyl curare observed after venom pretreatment.[4, 9]

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It appeared desirable to obtain additional information about the specificity of the structural permeability barriers and to determine the extent of penetration of several types of acetylcholinesterase (ACh-esterase) inhibitors into the axoplasm, with and without snake venom treatment.

MATERIALS AND METHODS

The dissection of the squid giant axon, exposure to venom and/or other agents, and extrusion of the axoplasm has been described,[4] as has the recording of electrical activity by means of external electrodes.[6] Since the compounds tested were not available in labeled form, we have measured the inhibition of a known cholinesterase (Ch-esterase) sample produced by the axoplasm of axons exposed to inhibitors. Because of of the small amount of material available, the magnetic diver gasometric method[10–12] was used for Ch-esterase measurements.

The giant axons plus many adhering small nerve fibers were first exposed for 30 min to either normal sea water or to a solution of 25 or 100 μg cottonmouth moccasin venom/ml sea water. After about 15-min rinsing in normal sea water the axons were exposed to neostigmine for 1 hr, physostigmine for 30 min, or paraoxon for 15 min; the axoplasm was then extruded. Subsequently, extruded axoplasm was first sucked into a calibrated Holter's braking pipette,[10] diluted with sea water to various degrees, and then mixed with Ch-esterase of whole blood of known activity. After being in contact for 20 min, this solution of axoplasm and enzyme was mixed with substrate–bicarbonate solution, introduced into the ampulla, and Ch-esterase activity measured with a magnetic diver device.[10–12] The volume of the reaction mixture was determined by weighing on a microbalance the empty ampulla and then the same ampulla with the charged reaction mixture. The concentrations of inhibitors in various dilutions of axoplasm were then estimated according to the calibration curves obtained with the same enzyme preparation and known inhibitor concentrations.

From the final dilution of axoplasm in the ampulla and from the externally applied concentration of inhibitors, the per cent penetration of inhibitors was calculated.

The ampullae used in this work had a fluid charge ranging from 0·6–1·0 μliter and a gas bubble of 0·8–1·2 μliter. The final concentration of ACh was 0·005 M; bicarbonate 0·016 (+ 5% CO_2). The final dilution of blood was 1:160.

Lyophilized venom from *Agkistrodon p. piscivorus* (cottonmouth moccasin; lot 6/29/61) was purchased from Ross Allen Reptile Institute, Silver Springs, Fla.

RESULTS

Cottonmouth venom has neither Ch-esterase activity nor does it inhibit the enzyme,[13, 14] therefore no correction is necessary for venom which may have penetrated into the axoplasm. In several experiments it was also observed that axoplasm from axons unexposed to inhibitors of Ch-esterase neither inhibits blood Ch-esterase nor contributes to its activity even when used 1:4, which was the smallest dilution employed in these studies.

In confirmation of previous reports,[6, 12] neostigmine had little effect on conduction even in venom-treated axons. Six axons were exposed to 5×10^{-2} M neostigmine for 1 hr, then to 25 μg cottonmouth venom/ml for 30 min, which had no effect on electrical activity, followed by neostigmine again for another hour. In no case did neostigmine cause more than a 15 per cent decrease in spike height in 1 hr.

Table 1 shows that neostigmine neither penetrates into controls nor into axons pretreated with 25 μg venom/ml. The lack of effect of neostigmine on conduction in these particular experiments is also shown in the table. Significant penetration of neostigmine is observed only after venom pretreatment of 100 μg/ml, a concentration which irreversibly blocks conduction.

TABLE 1. PENETRATION OF NEOSTIGMINE, PHYSOSTIGMINE, AND PARAOXON INTO AXOPLASM OF SQUID AXON

Per cent penetration = inside concentration expressed as per cent of the outside concentration. Venom = cottonmouth moccasin venom; 100 μg venom/ml itself blocked electrical activity. AP = action potential.

Compound	Pretreatment							
	None			Venom, 25 μg/ml			Venom, 100 μg/ml	
	Penetration (%)	Decrease AP (%)	No. of expts.	Penetration (%)	Decrease AP (%)	No. of expts.	Penetration (%)	No. of expts.
Neostigmine 5×10^{-2} M	0·02–0·08	0–20	5	0·02–0·1	0–20	8	0·5–1·0	6
Physostigmine								
$2{\cdot}5 \times 10^{-3}$ M	30–90	30–60	4	30–90	100*	2		
1×10^{-5} M	50–80	0	4	50–90	0	2		
Paraoxon								
1×10^{-2} M	5–6	100*	3	3–4	100†	3		
1×10^{-3} M	2–20	0–30	7					

* Block of conduction was reversible.
† Block of conduction was irreversible.

It is evident from Table 1 that physostigmine and paraoxon penetrate through axonal membranes and that the inside concentration of both inhibitors is high enough completely to inhibit cholinesterase in solution. However, only 10^{-2} M paraoxon and $2{\cdot}5 \times 10^{-3}$ M physostigmine caused a marked decrease in the conducted action potential (Table 1). These effects on electrical activity are in agreement with earlier findings.[7, 8, 19] For any of the particular experimental condition shown in Table 1 there was no significant correlation between the ranges observed for per cent penetration and per cent decrease in action potential; i.e. the experiment showing the lowest penetration did not necessarily show the least effect on the conducted action potential or vice versa. Probably because of the relatively high rate of penetration of physostigmine and paraoxon through the untreated axonal membrane, no further increase by venom pretreatment was observed.

In a group of experiments not listed in the table, axons exposed to physostigmine or paraoxon were washed in sea water for various lengths of time (5–20 min) before the axoplasm was extruded. The decrease of the concentrations of inhibitors found in the axoplasm after washing was to some extent proportional to the time of exposure to sea water. This indicates that these inhibitors can diffuse through the membrane in both directions.

DISCUSSION

The results demonstrate that neostigmine is unable to penetrate to any significant extent through the membranes of either control or axons pretreated with venom (25

μg/ml). The actual apparent penetrations of neostigmine observed under these conditions are respectively about 1/10 and 1/100 of those observed for ACh, choline, and dimethylcurare.[4] In addition, and as discussed previously,[4, 9] very low levels of apparent penetration may not represent actual penetration. Exceedingly slight contamination during the process of extrusion could account for the apparent penetration observed. It is especially significant that 25 μg cottonmouth venom/ml did not at all increase this low base level of observed penetration. This is in marked contrast to the findings with ACh, choline, and dimethylcurare where 15 μg cottonmouth venom/ml markedly increased their penetration.[4] These results might explain why 15 or 25 μg venom/ml renders the squid axon sensitive to the action of ACh and curare, but not to neostigmine.[7] Significant penetration of neostigmine is observed only after venom pretreatment of 100 μg/ml, which by itself will block electrical activity. The per cent penetration even under these conditions in which the plasma membrane itself must be severely damaged is, however, lower than those observed with ACh, choline, and dimethylcurare after pretreatment with 50 μg venom/ml.[4]

Poor penetration of neostigmine appears to be in good agreement with its ineffectiveness in blocking electrical activity. However, it is not so in regard to the other two cholinesterase inhibitors tested here. Physostigmine (10^{-5} M) and to a lesser extent paraoxon (10^{-3} M) readily penetrate from the surrounding fluid, through the axonal membrane, and into the axoplasm; yet they affect electrical activity only when applied in higher concentrations ($2{\cdot}5 \times 10^{-3}$ and 10^{-2} M respectively).

To aid interpreting and understanding these results it is helpful to recall the evidence in support of the essentiality of the ACh system in conduction, since the theory provides a possible explanation for our findings, and our findings add further details to the mechanism of action of this system in conduction. The ACh system is present in all conducting fibers,[1, 15] has special properties required for the assumption that it is responsible for the ionic permeability changes (e.g. a very small turnover time[16]), is localized within the conducting membrane,[17] and is inseparably associated with electrical activity.[15, 18, 19]

In addition, as mentioned above, ACh, curare, and other lipid-insoluble quaternary nitrogen compounds do affect conduction, provided they are able to penetrate.

Many lines of evidence indicate, and indeed the ACh theory would require, that the system is well organized within or adjacent to the excitable membranes, possibly in particles such as have been increasingly found for other multienzyme systems. Such an organization would most readily account for the high efficiency, precision, and speed of the permeability changes taking place in a few millionths of a second.

The lack of effect of these cholinesterase inhibitors on conduction, even though they penetrate into the axoplasm, may be attributed to many additional and at present poorly understood factors prevailing in an organized structure, such as a barrier to penetration not simply at the level of the whole axonal membrane but at specifically arranged structures protecting the active sites from extracellularly applied substances. To test this hypothesis critically it would have been interesting to measure the Ch-esterase of the squid giant axon membrane in presence of inhibitors. The relatively low enzyme activity in the finely dissected membrane[12] makes such measurement, even with the highly sensitive magnetic diver method, unfeasible since, for example, it would not be possible accurately to determine small amounts of Ch-esterase activity remaining after use of an inhibitor. It is also possible that proteins, such as enzymes when bound

in situ as a part of the membrane structure might have different binding constants for various drugs than when they are in solution.[20] Therefore it is possible that ACh-esterase in a living structure might be less sensitive to inhibitors than one would expect from *in vitro* experiments.

Previous studies using the giant squid axon have shown that the Ch-esterase reactivator 2-pyridine aldoxine methiodide can restore electrical activity which has been blocked by the Ch-esterase inhibitors DFP and paraoxon.[19] These experiments indicate the essentiality of Ch-esterase for conduction and appear to exclude the possibility that ACh may be eliminated and conduction restored by mechanisms other than enzymic hydrolysis, such as diffusion of ACh from receptor sites to surrounding structures, as has been previously suggested on theoretical grounds.[21, 22]

While we cannot be sure which of, and to what extent, the above hypotheses reflect *in vivo* conditions, it appears more reasonable to attribute the high and greatly varying concentrations of ACh, curare, neostigmine, physostigmine, paraoxon, etc. required for effecting electrical activity of various preparations to structural factors rather than to postulated differences in basic mechanisms. The latter conclusion would imply the assumption of a nearly infinite number of variations of basic chemical reactions. It would find some justification if even high concentrations of these specific inhibitors were unable to produce the postulated effect, but the relatively high concentrations required do not contradict the concept of the biochemical similarity of the basic mechanism. On the contrary, this view has found strong support by the various observations in which the concentrations required for blocking conduction have been decreased by factors ranging from 10 to 1,000 simply by removing inactive material such as sheaths or surrounding nerve bundles or by chemical pretreatment of nerves.

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REFERENCES

1. D. NACHMANSOHN, in *Symposium on New Perspectives in Biology*, ed. M. SELA, B.B.A. Library, vol. 4, p. 176. Elsevier, Amsterdam (1964).
2. T. H. BULLOCK, D. NACHMANSOHN and M. A. ROTHENBERG, *J. Neurophysiol.* **9**, 9 (1946).
3. M. A. ROTHENBERG, D. B. SPRINSON and D. NACHMANSOHN, *J. Neurophysiol.* **11**, 111 (1948).
4. P. ROSENBERG and F. C. G. HOSKIN, *J. gen. Physiol.* **46**, 1065 (1963).
5. R. R. WALSH and S. E. DEAL, *Amer. J. Physiol.* **197**, 547 (1959).
6. P. ROSENBERG and S. EHRENPREIS, *Biochem. Pharmacol.* **8**, 192 (1961).
7. P. ROSENBERG and T. R. PODLESKI, *J. Pharmacol. exp. Ther.* **137**, 249 (1962).
8. P. ROSENBERG and T. R. PODLESKI, *Biochim. biophys. Acta* **75**, 104 (1963).
9. F. C. G. HOSKIN and P. ROSENBERG, *J. gen. Physiol.* **47**, 1117 (1964).
10. M. BRZIN, M. KOVIC and S. OMAN, *C.R. Lab. Carlsberg, Ser. Chim.* **34**, 407 (1964).
11. M. BRZIN and E. ZEUTHEN, *ibid.*, p. 427.
12. M. BRZIN, W.-D. DETTBARN, P. ROSENBERG and D. NACHMANSOHN. In preparation.
13. E. A. ZELLER, *Helv. chim. Acta* **32**, 94 (1949).
14. P. ROSENBERG and W.-D. DETTBARN, *Biochem. Pharmacol.* **13**, 1157 (1964).
15. D. NACHMANSOHN, *Chemical and Molecular Basis of Nerve Activity*. Academic Press, New York (1959).
16. H. C. LAWLER, *J. biol. Chem.* **236**, 2296 (1961).
17. R. J. BARRNETT, *J. cell. Biol.* **12**, 247 (1962).
18. W.-D. DETTBARN, P. ROSENBERG and D. NACHMANSOHN, *Life Sci.* **3**, 55 (1964).

19. P. ROSENBERG and W.-D. DETTBARN. In preparation.
20. E. KATCHALSKI, *Harvey Lect. Ser.* **59** (1964). In press.
21. H. G. OGSTON, *J. Physiol. (Lond.)* **128**, 222 (1955).
22. N. EMMELIN and F. C. MACINTOSH, *J. Physiol. (Lond)*. **131**, 477 (1956).