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RELATIONSHIP OF ACETYLCHOLINESTERASE ACTIVITY TO AXONAL CONDUCTION*

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Abstract—We overcame previous difficulties in correlating electrical and acetylcholinesterase (AChE) activity, after exposure to organophosphate inhibitors, by using the intact squid giant axon, acetyl-β-methylcholine as substrate and a sensitive radiometric assay to measure AChE activity.

Reversible conduction block by a 6-min exposure to 10^{-2} M diisopropylfluorophosphate (DFP) was associated with 80 per cent inhibition of enzyme activity, while a 30-min exposure caused irreversible block and 99 per cent inhibition. Exposure to 5×10^{-2} M DFP for 1–5 min caused irreversible conduction block and 70 per cent enzyme inhibition. Pyridine aldoxime methiodide (PAM) restored electrical function in about one-half of the axons "irreversibly" blocked by DFP. Even in those axons in which PAM did not restore electrical activity, 30–50 per cent of the original AChE activity was present. The tertiary analogue of phospholine (TP) had no effect on electrical activity, although it inhibited 98–99 per cent of AChE. After cottonmouth venom pretreatment, TP blocked conduction in one-half of the axons and caused 80 and 93 per cent inhibition of AChE in the conducting and nonconducting axons respectively. There does not appear to be a simple and direct relationship between electrical and AChE activity.

FOR OVER 20 years, attempts to determine the acetylcholinesterase (AChE) activity in nerve fibers, after exposure to cholinesterase inhibitors, and to correlate these findings with nerve electrical activity have not been completely successful.[1,2] In some studies,[2,3] the enzyme activity was determined in homogenized tissue with reversible inhibitors, a procedure inadequate because a large fraction of the inhibitor is concentrated in the extracellular space. When the nerve preparation is not washed, the inhibitor in the extracellular space inhibits the intracellular enzyme during the process of homogenization. If the preparation is washed, both extra- and intracellular fractions of the inhibitor are removed and the activity is restored. When "irreversible" organophosphorus cholinesterase inhibitors are used, there is a release of entrapped inhibitor at the time of tissue homogenization, resulting in excess inhibition.[4–7] Because of these limitations, the previous attempts to correlate electrical and AChE activities on homogenized tissue are subject to considerable uncertainty.[1–16]

The determination of the enzymic activity in intact axons therefore appears preferable; however, this procedure also has limitations. The use of an intact axon preparation requires the use of a substrate specific for AChE that penetrates readily to all of the axolemmal enzyme thereby providing a true measure of total enzymic activity. In addition, only single axons can be used in the studies because of the

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uncertainties both as to numbers of fibers conducting and the actual enzyme activity in any particular fiber when multifibered preparations are used.[1] Previous data on the squid axon, a single fiber preparation,[17] indicated that acetylcholine could not reach all of the enzyme in the intact preparation. In a multifibered preparation, the vagus nerve bundle of the rabbit, acetyl methylcholine was found to penetrate to the enzyme much better than acetylcholine;[1] in addition, this substrate is also more specific for AChE than acetylcholine.[18]

We have now determined that the use of acetyl-β-methylcholine as a substrate permits the evaluation of the particular level of AChE activity required for electrical activity of intact squid axons previously exposed to cholinesterase inhibitors. Because of the relatively low AChE activity of this preparation,[17] it was necessary to use a highly sensitive and reliable radiometric technique for these assays. These studies are also of relevance to a theory, developed by Nachmansolm,[19–21] to account for the permeability changes associated with bioelectric activity.

MATERIALS AND METHODS

Giant axons containing adhering small nerve fibers were dissected from the squid (*Loligo pealii*). The axon ends were tied off with string to avoid loss of axoplasm. The preparations were then exposed to various drug treatments (see Table 2) while electrical activity of the axon was being monitored with external electrodes.[22] The lower threshold and the all-or-none response of the giant axon permits recording of the electrical responses without interference from the small nerve fibers. The preparations were then carefully dissected free of all adhering small nerve fibers under continuously running sea water. The wet weight of each axon (plus string) was then determined (2–10 mg) and the preparation placed intact in 40 μl of sea water containing 0·4 μmole (0·01 M) of acetyl-1-^{14}C-*dl*-methylcholine (New England Nuclear). At least 5 mg axon was used in those experiments in which inhibition was marked. After incubation in a 500-μl microhomogenizer for 2·5 – 4 hr at room temperature (20–23°), the homogenizer was placed in dry ice to stop the enzymic reaction. The procedures for chromatographically separating the labelled unhydrolyzed substrate from the labelled product, acetic acid-1-^{14}C, are those described previously.[7] Subsequent to incubation, the axon strings were carefully dissected free from the axon and weighed (0·5–1·0 mg); this weight was subtracted from the axon weight previously recorded. Determinations of spontaneous hydrolysis of the substrate in the absence of tissue and of the enzymatic activities of control tissues in the absence of inhibitors (see Table 1) were conducted in a similar manner. To determine activity in homogenized axons, the strings were cut off immediately prior to weighing, and the axons were homogenized with a Potter–Elvehjem type of microhomogenizer in 30 μl of sea water plus substrate, the remaining 10 μl being used to wash down the pestle. In some experiments the axoplasm was carefully extruded into preweighed capillary tubing using a Tygon-coated roller.[23] After redetermining the weight, the contained axoplasm was mixed with the 40 μl of incubation mixture. The tissue remaining after extrusion of the axoplasm consisted of the axolemma, Schwann cell and associated connective tissue and is referred to as the "envelope".

Diisopropylfluorophosphate (DFP; Merck, Sharp & Dohme) was dissolved in sea water and adjusted to pH 7·0–7·4 with sodium hydroxide before use. The anion of the oxalate salt of the tertiary analogue of phospholine $(C_2H_5O)_2$ P (O) SCH_2CH_2N

$(CH_3)_2$ (217 AO; a gift from Dr. R. A. Lehman, Ayerst Laboratories), just prior to use was precipitated with calcium chloride, centrifuged and the pH of the supernatant adjusted with sodium hydroxide to 7·5.

RESULTS

As recorded in Table 1, there are no differences in the AChE specific activities of the intact, homogenized, or homogenized–frozen–venom-treated axons when acetyl-β-methylcholine is used as the substrate. The envelope has considerably more activity than the axoplasm. Because of weighing errors associated with the small weights of envelope (0·4–0·6 mg) and the very rapid drying of this tissue during weighing, the later determination is subject to considerable error.

The effects of inhibitors on the electrical and AChE activities of the intact axon are shown in Table 2. In agreement with earlier findings,[10,24] DFP at 10^{-2} M caused a reversible block of conduction in 6 min and an irreversible block in 30 min. Higher concentration of DFP (5×10^{-2} M) caused a rapid (within 1 min) and irreversible block of conduction. Block of conduction was considered irreversible if, after 30 min rinsing with sea water free of DFP, there was no evidence of return of electrical activity. The extent of enzymatic inhibition in experiments 1–3 seemed more related to time of exposure than to concentration of inhibitors. Pyridine-2-aldoxime methiodide (PAM), a specific reactivator of AChE activity,[25] caused a restoration of electrical activity in 6 of 12 axons previously blocked by DFP (experiments 4 and 5), a result similar to that previously reported on the squid axon.[26] While in all experiments there was evidence of partial restoration of enzymatic activity by PAM, there did not appear to be any difference in enzymic activity between the 6 axons in which electrical activity returned after PAM and the 4 axons in which PAM did not restore conduction. The tertiary analogue of phospholine (TP), when applied directly to the axon, had no effect on electrical activity (experiments 6 and 7). However, after exposure to 25 μg/ml of cottonmouth moccasin venom, which itself has no affect on the height of the conducted action potential, TP blocked conduction in 10 min in two of four experiments (experiment 8). The findings with TP, on electrical activity, are in agreement with earlier data.[27]

TABLE 1. ACETYLCHOLINESTERASE ACTIVITY OF THE SQUID GIANT AXON*

Description	No. of Expts	Activity (mμmoles/mg tissue/hr)
Intact axon	8	2·48 ± 0·40
Homogenized axon	4	2·49 ± 0·48
Homogenized, frozen, venom-treated axon†	5	2·51 ± 0·61
Envelope	3	3·44 ± 1·48
Axoplasm	3	0·14 ± 0·05

* The results are corrected for spontaneous hydrolysis of the substrate, acetyl-1-^{14}C-β-methylcholine (0·41 ± 0·02 mμmoles/hr, 4 experiments). Enzyme activities are expressed as means ± standard errors.

† The axons were homogenized, frozen and thawed several times, followed by treatment with 1 mg/ml of cottonmouth moccasin venom.

TABLE 2. EFFECT OF INHIBITORS ON ACETYLCHOLINESTERASE AND ELECTRICAL ACTIVITY OF THE SQUID AXON *

Expt. No.	Compound	Concn (M)	Exposure (min)	No. of expts	Electrical activity	Enzyme (mμmoles/mg/hr)	Activity (% of control)
1	DFP	10^{-2}	6	3	RB	0·43 ± 0·10	17
2	DFP	10^{-2}	30	3	B-IR	0·02 ± 0·02	1
3	DFP	5×10^{-4}	1–5	4	B-IR	0·75 ± 0·26	30
	DFP	5×10^{-2}	2–3	6	B-IR		
4	Followed by PAM	5×10^{-4}	20–30	2	B-IR-PAM	0·86 ± 0·03	34
	Followed by PAM	5×10^{-2}	20–30	4	RB-PAM	1·12 ± 0·32	45
	DFP	1×10^{-2}	20–30	6	B-IR		
5	Followed by PAM	5×10^{-2}	30	4	B-IR-PAM	1·10 ± 0·28	44
	Followed by PAM	5×10^{-2}	30	2	RB-PAM		
6	TP	10^{-2}	30	4	No B	0·05 ± 0·02	2
7	TP	10^{-3}	30	6	No B	0·03 ± 0·01	1
	CM	25 μg/ml	30	4	No B		
8	Followed by TP	10^{-2}	10	2	B-IR	0·19 ± 0·09	7
	Followed by TP	10^{-2}	10	2	No B	0·57 ± 0·16	23
9	TP	10^{-3}	180†	3		0·04 ± 0·01	2

* Enzyme activities are expressed as means ± standard errors. All values are corrected for spontaneous hydrolysis of the substrate, acetyl-1-1-^{14}C-β-methylcholine. Intact axons were used in all experiments. The control activity was 2·5 mμmoles/mg/hr (see Table 1). Abbreviations used: DFP, diisopropylfluorophosphate; PAM, 2-pyridine-aldoxime methiodide; TP, tertiary analogue of phospholine; CM, cottonmouth moccasin venom; B-IR, block of electrical activity (which is irreversible even after 30 min of washing in sea water); RB, reversible block of electrical activity; No B, no block of conduction, RB-PAM, reversal by PAM of inhibitor block of conduction; B-IR-PAM, no reversal by PAM of inhibitor block of conduction.

† Exposed to TP during 3-hr enzymic assay.

DISCUSSION

It is critical to these experiments that all of the axolemmal AChE be assayed in the intact tissue if a relationship is to be established between electrical and AChE activity. We have no way of differentiating in the intact preparation between axolemmal, axoplasmic and Schwann cell AChE. Therefore our experiments must meet the even more rigid requirement that all of the axonal enzyme be assayed. Previous studies indicated that in intact tissues and even in certain homogenized tissues permeability barriers exist which prevent the demonstration of maximal enzymic activity; these barriers can be obviated by freezing, thawing and exposure to cottonmouth moccasin venom.[26,28–30] Based on a comparison of the AChE activity of intact and homogenized squid axon (Table 1), there is no evidence of a permeability barrier to acetyl-β-methylcholine; however at 10–20 per cent difference in activity might not be detected, as the standard errors of our method are about this large.

Inhibition of AChE (Table 2) appeared to be related to the duration of exposure of the axon to DFP; hence an exposure to 10^{-2} M DFP for 30 min inhibited the enzyme more strongly than 5×10^{-2} M for 1–5 min. We also confirmed earlier findings[26] that PAM, a specific reactivator of organophosphate, inhibited AChE and restored electrical activity in some but not all of the axons, after apparent "irreversible" conduction block by DFP. It now was possible, for the first time, to determine the enzyme activity in those axons in which PAM either reversed or failed to reverse block of conduction by DFP. When AChE was inhibited 99 per cent by a 30-min exposure to 10^{-2} M DFP, PAM caused a significant reactivation (~43 per cent). In contrast, after 2–3 min of exposure to 5×10^{-2} M DFP, AChE was inhibited only about 70 per cent and PAM did not cause significant reactivation (3 and 14 per cent). It is therefore possible that approximately 60 per cent of the enzyme is inaccessible to PAM. There was no significant difference in enzyme activity between those experiments in which PAM restored electrical activity and those in which it did not.

It was sometimes observed, after blocking concentrations of DFP were used, that the axon was altered, as indicated by greater fragility, loss of axoplasm, and the development of opalescence. It appears that the relatively high concentrations of DFP or fluoride ion liberated as a result of DFP hydrolysis are affecting other membranal systems in addition to AChE. Almost a quarter century ago, Toman *et al.*[31] suggested that high concentrations of DFP might block conduction nonspecifically rather than by inhibiting AChE.

The inhibitor TP readily penetrates, in its inhibitory form, into the axoplasm of the squid axon[27] and is not detoxified nor hydrolyzed by tissue enzymes. Although not affecting electrical activity (Table 2) in the absence of venom pretreatment, TP at 10^{-2} and 10^{-3} M appeared to inhibit in 30 min 90–99 per cent of the total AChE activity. In contrast, after venom pretreatment, conduction was blocked in two experiments by a 10-min exposure to TP and AChE activity was 93 per cent inhibited. In two other experiments, after venom treatment, conduction was not affected by a 10-min exposure to TP and in these experiments AChE was 77 per cent inhibited.

These experiments appear to argue against a simple and direct relationship between electrical and AChE activity after exposure to organophosphates. The data suggest that electrical functioning of nerves may be maintained in the presence of very low AChE levels. If acetylcholine is involved in conduction, it is possible that, after cholinesterase inhibition, endogenous acetylcholine can be rapidly removed after

its interaction with the receptor by some means other than, or in addition to, enzymatic hydrolysis, perhaps by rapid diffusion over a short distance or by binding to other proteins or subcellular components near the receptor.

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