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REFERENCES

1. R. DUSCHINSKY, E. PLEVEN and C. HEIDLEBERGER, *J. Am. chem. Soc.* **79**, 4559 (1960).
2. S. S. COHEN, J. G. FLAKS, H. D. BARNER, M. R. LOEB and J. LICHTENSTEIN, *Proc. natn. Acad. Sci. U.S.A.* **44**, 1004 (1958).
3. K.-U. HARTMAN and C. HEIDLEBERGER, *J. biol. Chem.* **236**, 3006 (1961).
4. C. HEIDLEBERGER, *Prog. nucleic Acid Res.* **4**, 1 (1965).
5. P. REICHARD and O. SKÖLD, *Nature, Lond.* **183**, 939 (1959).
6. P. REICHARD, O. SKÖLD, G. KLEIN, L. REVESZ and P. -H. MAGNUSSON, *Cancer Res.* **22**, 235 (1962).
7. D. K. KASBEKAR and D. M. GREENBERG, *Cancer Res.* **23**, 818 (1963).
8. C. HEIDELBERGER, G. KALDOR, K. L. MUKHERJEE and P. B. DANNEBERG, *Cancer Res.* **20**, 903 (1960).
9. P. REYES, *Biochemistry* **8**, 2057 (1969).
10. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
11. D. ROBERTS, I. WODINSKY and T. C. HALL, *Cancer Res.* **25**, 1899 (1965).
12. A. B. HILL, *Principles of Medical Statistics*, p. 193. Oxford University Press, New York (1961).
13. R. G. HOFFMAN, *Statistics for Medical Students*, p. 98. CHARLES C. THOMAS, Springfield, Ill. (1963).
14. T. C. HALL, D. KESSEL, A. GODSILL and D. ROBERTS, *Proc. Am. Ass. Cancer Res.* **9**, 27 (1968).
15. O. SKÖLD, *Ark. Kemi* **17**, 59 (1960).
16. A. KORNBERG, in *Chemical Basis of Heredity* (Eds. W. D. MCELROY and B. GLASS), p. 579. Johns Hopkins Press, Baltimore (1957).
17. P. REICHARD and O. SKÖLD, *Acta. chem. scand.* **11**, 17 (1957).
18. D. HATFIELD and J. B. WYNGAARDEN, *J. biol. Chem.* **239**, 2580 (1964).
19. H. E. SKIPPER, *Cancer Res.* **25**, 1544 (1965).

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Action of acetylcholine in the presence of organophosphates on single axons of the lobster*

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THE GIANT axon of the circumesophageal connectives of the lobster is a suitable preparation for studying the effects of acetylcholine (ACh) on axonal conduction. It was shown previously that ACh depolarizes the conducting membrane, causing repetitive firing, then block of conduction. These actions seemed similar to those seen at the neuromuscular junction. However, not all of the findings

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supported this view. Reversible inhibitors of cholinesterase in concentrations which strongly inhibit the hydrolysis of ACh prevent the action of ACh rather than potentiate it. This depressant action appears not to be analogous to a curare action, since curare does not antagonize the effect of ACh on these axons.[1]

This paper reports studies of the direct action of irreversible cholinesterase inhibitors such as diisopropylphosphorofluoridate (DFP) and phospholine on resting and action potentials of giant axons and their effect on the ACh sensitivity before and after ACh-esterase reactivation by pyridine-2-aldoxime methiodide (2-PAM).

METHODS

The methods for the electrophysiological part of the paper were the same as those described previously.[1] The hydrolysis of ACh was measured microgasometrically using the magnetic diver technique with ACh 5×10^{-3}M as substrate.[2] For these measurements of cholinesterase activity, the giant axons were freed of all small nerve fibers. Sections of the cleaned axon of various lengths were dissected and linear dimensions determined with the microscope, using a calibrated eyepiece.

Cholinesterase was measured either in intact fragments of axonal cylinder or in homogenized samples of inhibitor-treated or untreated preparations. The cleaned axons were ground in a small glass homogenizer (50 μl).

The compounds used were ACh, diisopropylphosphorofluoridate (DFP), *o*, *o*-diethyl S-(2-trimethylammoniumethyl) phosphorothioate (phospholine), and pyridine-2-aldoxime methiodide (2-PAM).

TABLE 1. EFFECTS OF CHOLINESTERASE INHIBITORS ON ELECTRICAL AND ENZYME ACTIVITY OF SINGLE AXON*

Inhibitor conc (M)	Time of exposure (min)	Time of washing (hr)	Hydrolysis of ACh (%) Intact	Hydrolysis of ACh (%) Homogenized	Effects on electrical activity
Phospholine					
1×10^{-3} (2)	30	8–12	< 3†	< 3	No effect
1×10^{-6} (1)	30	10	< 3	< 3	No effect
DFP					
$2{\cdot}5 \times 10^{-2}$ (3)	15–30	5–9	< 3	< 3	Reversible depolarization and block
1×10^{-2} (4)	15–40	12–31		< 3	Reversible depolarization and block, repetitive firing
5×10^{-3} (1)	35	8	4		None
1×10^{-3} (2)	35	3–7	4–8		None
1×10^{-3} (1)	35	27		4	None
1×10^{-4} (2)	20	29–31		55	None
5×10^{-2}	30				Irreversible block and depolarization
$+ 1 \times 10^{-3}$ 2-PAM (1)	15	9	80		

*Enzyme activity of isolated axons was measured microgasometrically and electrical activity was monitored with an intracellular microelectrode. After exposure to the inhibitor, axons were washed for varying lengths of time and then checked for electrical and enzymic activity. The cholinesterase activity is given as activity in per cent of control. Number of experiments in parentheses.

†The lower limit of the method is 3 %.

RESULTS

Phospholine at a wide range of concentrations did not affect the action potential or membrane potential of isolated giant axons; but, as can be seen in Table 1, a concentration as low as 1×10^{-6}M seems to inhibit about 97 per cent of the accessible cholinesterase. There was no difference between intact and homogenized axons. However, intact axons may not indicate all the enzyme, but only that part accessible in an intact structure. A 10,000-fold higher concentration of phospholine still has no effect on either resting or action potential of the axons; however, it repolarizes the membrane and restores the conducted spike in the presence of ACh, which caused depolarization and reduction of the action potential. As can be seen from Fig. 1, phospholine (1×10^{-6}M) applied to the axon for 30 min can irreversibly prevent the action of 1×10^{-2}M ACh. After 35 min of exposure to ACh, there is no change in amplitude or shape of the action potential. Even after prolonged washing, the choli-

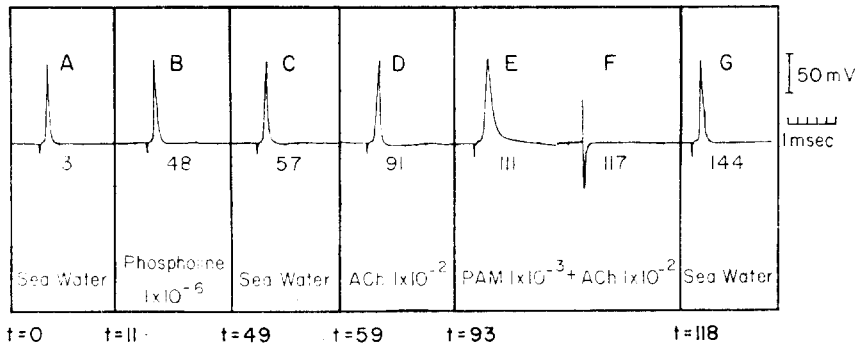

FIG. 1. Effects of ACh, phospholine and PAM on the action potential. The numbers beneath the action potentials are the times at which pictures were taken; t equals absolute time of experiment at which solutions were changed. A, Control spike in sea water, B, spike after 37 min in phospholine (10^{-6}M) no effect on the action potential is seen; C, return to sea water; D, after 30 min in ACh (1×10^{-2}M), no effect on the action potential can be detected; E and F, 24 min in PAM (1×10^{-3}M) and ACh; block of conduction is seen which is reversed in sea water, G.

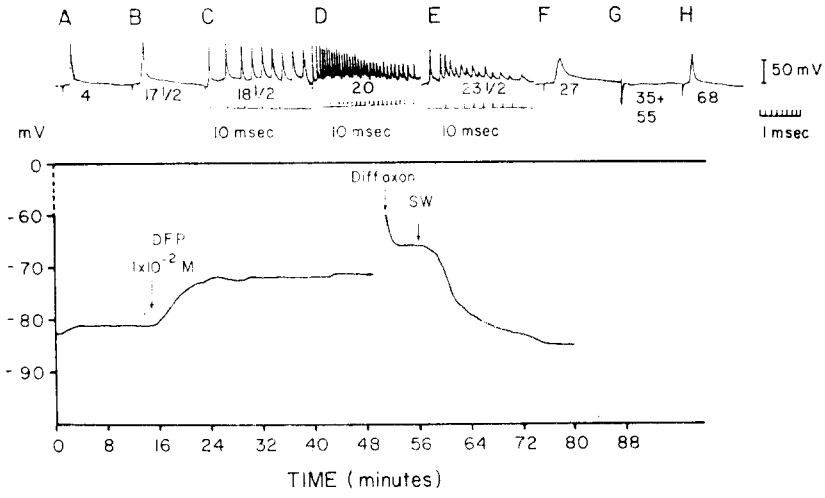

FIG. 2. Repetitive firing caused by DFP (1×10^{-2}M). In this and the following figure the top part shows photographs of the action potentials, the bottom part tracings of the resting potential. The numbers below the action potential correspond to the time scale of the abscissa. Changes of solution are indicated by arrows. pH, 7·8; T 23°; SW, sea water. With the onset of a 10 mV depolarization, repetitive firing is seen (C, D, E) followed by block of conduction (G). There is no continuous depolarization. On return to SW (H), the membrane repolarizes and the action potential recovers. A different axon of the same bundle has been impaled a second time. A reversible depolarization and block of conduction are apparent. Different time calibration for C, D, E as indicated; each division corresponds to 10 msec.

nesterase remains inhibited by phospholine (1×10^{-6}M) in intact and homogenized axons (Table 1). When 2-PAM (1×10^{-3}M) is added to the ACh, a reversible block of conduction occurs. 2-PAM by itself did not affect electrical activity and did not interfere with the ACh action.

The tertiary analog of phospholine, which is more lipid-soluble than the quaternary phospholine, has a similar action and potency.

As seen in Table 1, DFP seems to be a weaker inhibitor of lobster cholinesterase than phospholine. A possible explanation for this is the presence of DFP-ase, which has been found in the squid and lobster giant axons.[3] The activity of this enzyme in lobster nerve is only 10 per cent of that of squid. In spite of this difference, the direct effect of DFP on electrical activity on the lobster axons is seen at the same concentration as on the squid giant axon.[3] Only at high concentrations (1×10^{-2}M to 5×10^{-2}M) are the action and membrane potentials affected. DFP (5×10^{-2}M) depolarizes the membrane by 60 mV in 5 min and blocks the action potential within 1 min. On return to sea water, there is a small transient recovery of the membrane potential which is followed by a slow continuous depolarization. 2-PAM (1×10^{-3}M) does not repolarize the membrane, but it reactivates the cholinesterase activity to its initial level (Table 1).

The cholinesterase activity is blocked irreversibly after the axon has been exposed to DFP ($2{\cdot}5$ 10^{-2}M) for 15 min (Table 1). The axon is depolarized by 12 mV in a short time and the action potential is blocked. The resting potential as well as the action potential recovers slowly in sea water. The enzyme remains inhibited even after washing in sea water for 31 hr (Table 1). Figure 2 shows the effect

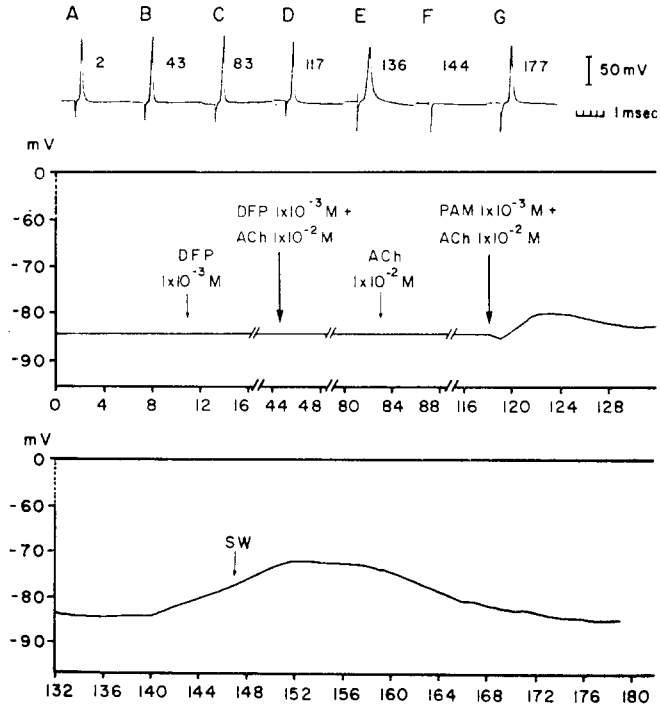

FIG. 3. The effect of DFP and PAM on the ACh action. The control action potential in SW (A). After 40 min in DFP (10^{-3}M), no effects on the action potential (B) or resting potential are seen. This pretreatment with DFP (10^{-3}M) inhibits the action of ACh (10^{-2}M) even after the DFP is removed from the bathing solution (C, D). Addition of PAM (1×10^{-3}M) to the ACh (1×10^{-2}M) causes ACh to depolarize the membrane and block conduction (E, F). Return to SW causes repolarization of the membrane and restores conduction (G). PAM by itself has no effect on the membrane or action potential.

of DFP (1×10^{-2}M) on electrical activity. A small depolarization (9 mV) is seen. During the initial depolarization, repetitive firing occurred with a maximal frequency of 150/sec. The action potentials decrease in amplitude toward the end of the train of repetitive firing and block of conduction is seen after 20 min in DFP. Impalements of a different axon show a steady state potential and block of conduction. After return to sea water, the resting and action potentials recover, while the cholinesterase activity is still inhibited (Table 1).

After 35 min of incubation with 1×10^{-3}M DFP, there remains a residual cholinesterase activity in the intact axons of 6–8 per cent and of 4–5 per cent in the homogenized axons (Table 1). While there is no effect on electrical activity in 35 min (Fig. 3), DFP prevents the action of ACh. After the addition of 2-PAM (1×10^{-3}M), an immediate effect of ACh on the resting potential can be observed. A small initial depolarization occurs, followed by a repolarization and a second slower depolarization. The action potential, as was seen repeatedy, increases in duration and is blocked at the beginning of the second depolarization. After 30 min in sea water, a recovery of resting and action potentials is observed.

DFP (1×10^{-4}M) initially causes a small hyperpolarization of the axonal membrane which is accompanied by an increase in spike amplitude. If ACh (1×10^{-2}M) is added to the DFP solution, the membrane depolarizes slowly without reaching a steady state. The action potential is not blocked after 45 min in ACh; it decreases in amplitude to less than 50 per cent of its initial value and the duration is increased. There is a fast recovery in sea water of both the action and resting potentials. Under these conditions, DFP inhibits cholinesterase activity of a homogenized axon only 50 per cent (Table 1).

DISCUSSION

Concentrations of irreversible inhibitors of the enzyme which do not affect electrical parameters of the axons have one characteristic effect in common: they inhibit irreversibly the action of ACh on membrane and action potentials. Axonal enzyme that has been inhibited by either DFP, phospholine or paraoxon shows no recovery of activity after prolonged washing (18–22 hr) in sea water. DFP and phospholine, as well as other alkylphosphates, inactivate cholinesterase by phosphorylating the esteratic site.[4] This reaction is only slowly or not at all reversed *in vitro*, unless a nucleophilic reagent is added, but reactivation may be much faster in the tissue, as more and more reports indicate, possibly due to nucleophilic groups present in the membrane. The most effective of the nucleophilic agents is 2-PAM, which is highly specific for ACh-esterase.[5,6] However, at a concentration of 1×10^{-3}M, used in the experiments reported above, other cholinesterase, at least in solution, may be reactivated also. Most of the enzyme in the axons is, however, ACh-esterase. In our experiments, when 2-PAM was used to reactivate ACh-esterase activity in the lobster axons, a concomitant reestablishment of the action of ACh on membrane and action potentials was observed. An additional indication that the action of ACh observed in these experiments depends on the presence of active ACh-esterase can be found in the experiment using lower DFP concentrations, which only partially inhibit the enzyme. In the axon with about 50 per cent of its original enzyme activity remaining, the action of ACh was also only partially inhibited.

Irreversible depolarization caused by DFP (5×10^{-2}M) is possibly due to a nonspecific structural damage to the membrane. The axoplasm and the axonal envelope appeared to be deteriorating as seen under the microscope. The depolarization could not be reversed by 2-PAM, even though the enzyme activity was fully restored (Table 1).

The findings reported in this paper strongly support the conclusion of similar experiments with reversible inhibitors which were published recently.[1] It was suggested that in this prepatation the action of ACh is due to a strong acidification. The depolarization by ACh and its effect on the action potential are closely associated with hydrolytic activity of cholinesterase. The splitting of ACh generates hydrogen ions within or at the axonal membrane, causing a localized change in pH. This shift of pH may be responsible for the observed electrophysiological response to ACh. Therefore, the externally applied ACh apparently has in this case only an indirect effect on the axonal membrane. However, these conclusions apply only to the axons of the circumesophageal connectives of the lobster; analogous effects were not observed on other preparations.[7]

A problem which still has to be discussed is that of the meaning of the cholinesterase determination in our experiments. As we and many other investigators have become increasingly aware, there is at present no method to evaluate quantitatively the remaining cholinesterase activity after a nerve

has been exposed to organophosphate. In homogenized nerve, traces of organophosphate may have been trapped which remain even after washing. It has been shown that even with organic solvents it is impossible to remove the organophosphates completely. Moreover, even homogenized fibers may not give the total value of the enzyme in the membrane. Since it seems that the proteins in the membrane form lipoprotein complexes, the lipid being on the outside,[8] it is thus entirely possible that some poorly lipid-soluble compounds, especially when charged, are unable to reach the active sites in the structurally highly organized membranes. Even in submicroscopic particles, part of the active enzyme might be protected and inaccessible to ACh (e.g. myelinated fibers homogenized).[9] Intact structures are even more questionable, since we know that homogenization may increase cholinesterase activity of axons 200–300 per cent.[10] Thus the data presented here may give a distorted picture of the cholinesterase activity at the end of the experiment.

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REFERENCES

1. W-D. DETTBARN and E. BARTELS, *Biochem. Pharmac.* **17**, 1833 (1968).
2. M. BRZIN, W-D. DETTBARN, P. ROSENBERG and D. NACHMANSOHN, *J. Cell. Biol.* **26**, 353 (1965).
3. F. C. G. HOSKIN, P. ROSENBERG and M. BRZIN, *Proc. natn. Acad. Sci. U.S.A.* **55**, 1231 (1966).
4. D. E. KOSHLAND, JR., *Adv. Enzymol.* **22**, 45 (1960).
5. I. B. WILSON and S. GINSBURG, *Biochim. biophys. Acta* **18**, 168 (1955).
6. H. KEWITZ, I. B. WILSON and D. NACHMANSOHN, *Archs. Biochem. Biophys.* **64**, 456 (1956).
7. W-D. DETTBARN, *Life Sci.* **12**, 910 (1963).
8. E. P. KENNEDY, *Proc. Plenary Sessions of the Seventh Int. Cong. Biochem.* I.U.B., vol. 36, p. 51 (1967).
9. F. C. G. HOSKIN and P. ROSENBERG, *J. gen. Physiol.* **47**, 1117 (1964).
10. P. ROSENBERG and W-D. DETTBARN, *Biochem. Pharmac.* **13**, 1157 (1964).