ELECTRICAL ACTIVITY EVOKED BY A SPECIFIC CHEMICAL REACTION

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SUMMARY

The aim of the investigations has been the demonstration that electrical activity, irreversibly blocked by the inhibition of acetylcholinesterase with organophosphorus compounds, may be restored by a specific chemical reaction, viz. by reversing the process with a specific reactivator which displaces the phosphoryl group from the enzyme.

1. Frog Sartorius muscle, dissected into two strips, was exposed to Paraoxon until irreversible block of electrical activity was reached. One strip was then exposed to the reactivator.

2. Pyridine-2-aldoxime methiodide (2-PAM), a powerful reactivator of phosphorylated enzyme, and several more lipid soluble derivatives did not restore electrical activity either in normal or in denervated muscle. Electrical activity could be evoked in strips in which it had been nearly completely abolished, with benzoyl-2- and -4-pyridine aldoxime methiodide. Nine out of eleven experiments were successful.

3. The inhibitor used was not quite suitable: the transition from irreversible block of electrical activity to a severe damage of the muscle, with gross alterations such as rigidity and decoloration, took place rather fast. The multifiber preparation used was for other reasons unfavorable for the purpose of the experiments. It is suggested that with other organophosphorus inhibitors and other types of preparation a still better demonstration would be possible that electrical activity may be evoked by a specific chemical reaction.

4. Frequently, the spike height and amplitude of the muscle, on exposure to either eserine or Paraoxon, were markedly increased before the decrease and eventual abolition. With Paraoxon the effect occurred sometimes in the recovery phase. These effects support the assumption that the rapid increase in sodium conductance during nerve activity is due to the action of acetylcholine and the rapid restoration to the normal level to that of acetylcholinesterase.

INTRODUCTION

The importance of the acetylcholine system for the generation of bioelectric potentials, which propagate impulses in the conducting membranes of nerve and muscle, has

Abbreviations: PAM, pyridine-2-aldoxime methiodide; PAD, pyridine-2-aldoxime dodecyl iodide; DFP, diisopropylfluorophosphate.

been demonstrated in many ways. The data have recently been summarized and evaluated in a monograph by NACHMANSOHN[1]. One essential point upon which the evidence is based, is the inseparability of conduction from the activity of acetylcholinesterase demonstrated with specific inhibitors on a great variety of conducting tissues. Under no condition has it been possible to dissociate electrical and enzyme activity in any type of conducting tissue: electrical activity invariably stops when the enzyme activity falls to about 20 % of the initial value.

The development of organophosphorus compounds, which are powerful irreversible cholinesterase inhibitors, their increasing application as insecticides, and their potential use as chemical warfare agents ("nerve gas") rendered the mode of the reaction of these compounds with the enzyme of great theoretical and practical interest. The elucidation of the precise mechanism has revealed that these compounds, when reacting with the enzyme, form a phosphorylated enzyme instead of the acetylated enzyme which is the intermediary form in the physiological process[2]. But whereas the acetylated enzyme reacts rapidly with water and leads to acetate and restored enzyme, the phosphoryl group can only be displaced from the enzyme by a chemical reaction[3]. One of the most powerful compounds specifically designed to reactivate the phosphorylated enzyme proved to be PAM[4]. The speed of reactivation is so high that PAM applied to animals proved to be an efficient antidote, especially combined with atropine[5,6].

It appeared of interest to test whether conduction irreversibly blocked by organophosphorus compounds could be restored by the reactivation of phosphorylated enzyme. In the affirmative case it would be a demonstration that the abolished electrical activity can be restored by a highly specific chemical reaction, *i.e.*, the reactivation of acetylcholinesterase. The significance of such an experiment is obvious. PAM is insoluble in lipids and could not be expected to reactivate the enzyme in conducting fibers since it is known that such type of compounds do not affect the conducting membrane[7,8]. Therefore, a modification of its structure consisting in a substitution of the methyl group by a dodecyl group was introduced: 2-PAD, making the compound very lipid soluble. However, when this compound was applied to conducting tissue, it proved to be a strong blocking agent of electrical activity: it depolarizes the conducting membrane and produces muscular contraction outside the synapse by direct action on the conducting membrane, thus acting to some extent as a lipid soluble analogue of acetylcholine[9-14]. In higher concentrations the action is difficult to reverse. Thus, although valuable in other respects, the compound was useless for the specific purpose for which it was originally designed.

In 1959, AXELSON AND THESSLEFF[15] reported an effect of acetylcholine on denervated muscle outside the neuromuscular junction. This ester, as well as other methylated quaternary ammonium compounds, is known to have no effect on the conducting membrane of normal muscle when applied externally. The new results seemed to indicate, therefore, that following denervation an increase in permeability to acetylcholine may take place so that now a fraction of the compound applied may reach the active site.

If denervation facilitates access of acetylcholine to the active sites in the conducting membrane, it may do the same for other quaternary ammonium ions, such as 2-PAM. In that case, reactivation of phosphorylated enzyme within the membrane and restoration of irreversibly blocked electrical activity seemed to be possible.

Experiments testing this assumption showed, however, that the increase in permeability is relatively small and, in any case, not adequate enough to produce the desired result. However, another derivative of PAM, benzoyl PAM, more lipid soluble than PAM but not quite as lipid soluble as PAD, proved to be more successful and the results obtained are described in this paper.

METHODS

Summer stock Rana pipiens were used. About 1 cm of the lumbosacral plexus on one side of the frog was removed under urethane anesthesia. The frogs were kept for one month or more to insure complete denervation before they were used for experiments. Since a change in permeability to various compounds was expected following denervation, the contralateral Sartorius muscle could not be used as control. Each Sartorius was therefore dissected longitudinally into about equal halves and one of these used as the control for the other. This procedure had the additional advantage that the strips were thinner than the whole muscle and the penetration of the compounds was, therefore, facilitated. The strips were kept in oxygenated Ringer for 1 h or more and were then mounted in the experimental chamber. Two platinum electrodes and a Grass stimulator were used for stimulation. Recordings were obtained by external platinum electrodes, a Grass AC Preamplifier and an oscilloscope. The records were photographed with a Grass camera. The composition of Ringer solution was 119 m*M* NaCl, 2.5 m*M* KCl, 1.8 m*M* $CaCl_2$, 2.5 m*M* $NaHCO_3$. The pH was frequently checked and kept within 7.3–7.5 range except when working with eserine, when a slightly more alkaline pH (about 7.8) was used. All experiments were done at room temperature.

The organophosphorus compound used was Paraoxon. About 30 experiments with this compound alone were performed in order to obtain the optimal concentration and the appropriate time of exposure needed to produce irreversible block of action potential without leading to severe damage to the muscle.

Reactivators tested were PAM, isonicotinoylformaldoxime methiodide, isonicotinoylformaldoxime methiodide, isonicontinoylformaldoxime (tertiary), 2-benzoylpyridine oxime methiodide and 4-benzoylpyridine oxime methiodide. All these compounds were synthesized by Dr. S. GINSBURG. Paraoxon was prepared by Dr. A. GOLD. The authors are greatly indebted to both.

RESULTS

Effects of eserine and Paraoxon

Eserine in concentrations of 1 to 7 μmoles/ml abolished the electrical activity, the response to direct stimulation, within 10 to 40 min. This inhibition was readily reversible. Although some difference was observed in the response of normal and denervated muscle, it was not striking. On several occasions block of electrical activity by eserine was preceded by a significant increase in the amplitude of the action potential (Fig. 1).

Paraoxon in 4 μmoles/ml also abolished electrical activity of normal and denervated muscle, but much faster than eserine. On short exposure to Paraoxon the inhibition was at first reversible, as described with other irreversible inhibitors, such

as for instance DFP, on a great variety of nerve fibers[1]. Irreversible inhibition of electrical activity was obtained with either increased concentration of the compound or by longer exposure. Too long exposure led to severe and irreparable damage grossly indicated by decoloration, contracture and rigidity of the muscle. Optimum

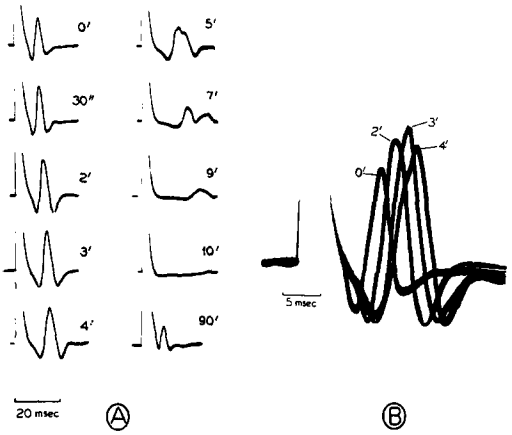

Fig. 1. Increase of the height of the action potential in a frog sartorius muscle on exposure to eserine. The muscle was exposed to $7.2 \cdot 10^{-3}$ M eserine salicylate for 10 min and 30 sec at pH 7.8. A. Records shown were taken at 0 time and after exposure at times indicated in the Figure. Stimulus artifact precedes action potential. Electrical activity was virtually abolished after 10 min. Partial recovery after 90 min. B. Same records superimposed. In the following figures only the superimposed records will be shown.

concentration of Paraoxon which blocked conduction without noticeable damage was found to be about 5–6 μmoles/ml and the duration of the exposure 8 to 15 min. The sensitivity to Paraoxon varied, however, greatly from muscle to muscle, while the two sections of the same muscle usually behaved in essentially the same way. Again, no marked difference was observed between the normal and the denervated muscle.

An increase in spike height and amplitude of muscle exposed to Paraoxon was observed in 13 different experiments, either during the initial period of exposure —quite similar to the eserine effect—or during the recovery phase in those muscles which were exposed to concentrations insufficient for blocking electrical activity irreversibly (Figs. 2 and 3).

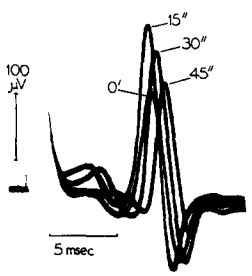

Fig. 2. Increase of spike height in a frog sartorius muscle on exposure to Paraoxon (1.5 mg/ml). Records shown (superimposed) were taken at 0 time and 15, 30 and 45 sec after exposure.

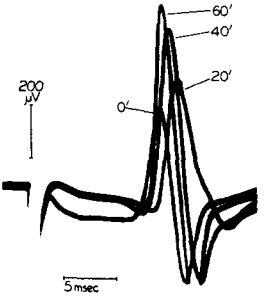

Fig. 3. Increase of the height of action potentia in denervated frog Sartorius muscle during recovery, after the muscle had been exposed to Paraoxon (1 mg/ml) for 10 min. Records shown (superimposed) were taken at 0 time and 20, 40 and 60 min after the start of the experiment.

Effect of antidotes on electrical activity of muscles exposed to Paraoxon

After a series of experiments with Paraoxon alone it became clear that it was only a very short step from an irreversibly inhibited preparation, which remained viable, to one which was irreparably damaged. It appeared that the relatively best procedure

was that of producing a condition in which the irreversible inhibition was not yet complete but would permit on washing with Ringer's solution a small spontaneous recovery of electrical activity indicating that with this concentration and time of exposure to Paraoxon the muscle remained in a relatively fair condition while most of the electrical activity remained suppressed by the specific action of the inhibitor. The improvement of the electrical activity obtained in the second half of the same muscle inhibited with the identical concentration and time of exposure to Paraoxon but exposed thereafter to an antidote could then be expressed in percentage of the control recovery. An improvement of 50 to 100 % over that without reactivator was considered a mild success, while an increase in the height of the action potential by a factor of 3 or more was accepted as significant. Those experiments in which the control muscle clearly showed irreparable damage due to overexposure were discarded. In one the exposure was not long enough so that the control muscle recovered fully. In five the recovery in the muscle strips treated with reactivator was very much greater than in the controls (Figs. 4 and 5). In two the improvement was by a factor of 2 to 3 and in two by a factor less than 2. Two experiments were failures. Thus out of 11 experiments 9 have shown improvement of electrical activity, in 5 of them this improvement was excellent, while only 2 failed to show improvement with the reactivator.

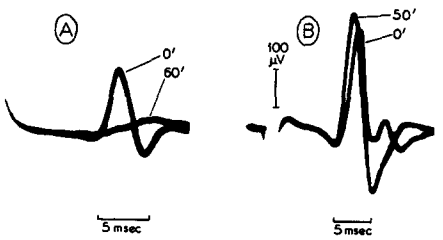

Fig. 4. Reappearance of electrical activity by the action of benzoyl-pyridine oxime methiodide in a muscle exposed to Paraoxon until irreversible block was achieved. A denervated frog sartorius muscle was dissected into two strips, both were exposed to 1.5 mg/ml of Paraoxon for 8 min. A, control. Action potential was abolished within 3.5 min. The strip was washed 3 times with Ringer's solution and then kept in that solution. Records shown were taken at 0 and 60 min. B, second strip was washed 3 times with Ringer's solution and then kept in 10^{-2} *M* 2-benzoylpyridine oxime methiodide from the 9th to the 45th min after the start of the experiment. Records shown were taken at 0 and 50 min. Records are superimposed.

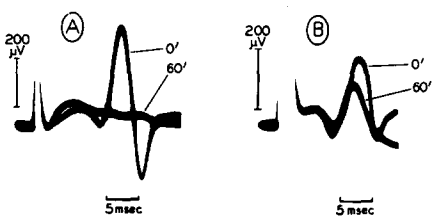

Fig. 5. Reappearance of electrical activity by the action of benzoyl-pyridine oxime methiodide in a muscle exposed to Paraoxon until irreversible block was achieved. A normal frog sartorius muscle was dissected into two strips; both were exposed to 1.5 mg/ml of Paraoxon for 8 min. Action potentials in both strips disappeared within 2 min. A. Control. Washed twice with Ringer's solution and then kept in that solution. Records shown were taken at 0 and 60 min. B. Second strip was washed twice with Ringer's solution and kept in 10^{-2} *M* 2-benzoyl-pyridine oxime methiodide from the 9th to the 30th min after the start of the experiment, then returned to Ringer's solution. Records shown were taken at 0 and 60 min.

DISCUSSION

Although extensive investigations have established that electrical activity in axons fails when acetylcholinesterase activity falls below a certain level, about 20 % of the initial value, the result was challenged by DEL CASTILLO AND KATZ[16] on the basis that the concentrations used are "unconventionally" high when compared with the concentrations used at synaptic junctions. The authors implied an unspecific action of the inhibitors. The high concentrations are, however, necessary because axons are

surrounded by structural barriers. Moreover, the assumption of an unspecific effect is difficult to reconcile with the various demonstrations that different types of chemical compounds used at different concentrations under a variety of conditions invariably block conduction at the same level of esterase activity. The time of exposure required may differ greatly but the percentage of activity essential for conduction is the same. Recently, DETTBARN applied eserine to single Ranvier nodes of single fibers of frog sciatic nerve and found the effectiveness of this compound to block conduction increased by a factor of 1000 (see ref. 12). In this preparation, as recent electron microscope pictures have revealed, the conducting membrane is only poorly protected by a thin and apparently porous structure[17].

Obviously, the inseparability of electrical and enzyme activity would gain strong additional support by the demonstration that irreversibly blocked electrical activity may be evoked again by the specific reactivation of acetylcholinesterase. PAM has been designed to reactivate specifically phosphorylated acetylcholinesterase. It has been shown to have molecular complementarity to the phosphorylated enzyme[18]. For other phosphorylated esterases PAM is a relatively poor reactivator. This compound would, therefore, be suitable for the demonstration desired except for its lipid insolubility preventing it from reaching the active site. The expectation that permeability to lipid insoluble quaternary compounds would sufficiently increase in denervated muscle fibers did not materialize. Among the modifications of PAM making the molecule more lipid soluble, so far only benzoyl 2- and 4-PAM proved to be effective. It was possible to evoke good electrical activity after exposure to the reactivator in muscle fibers in which electrical activity was nearly completely and irreversibly abolished.

The experiments are not yet fully satisfactory. Out of 11 experiments 2 were failures. It would be desirable to obtain experimental conditions where completely and irreversibly blocked fibers would regain normal electrical activity. Various factors are unfavorable in the experiments performed. The material used is a multifiber preparation. Both simultaneous inhibition and simultaneous reactivation of all the conducting membranes appear unlikely since the penetration is relatively slow and the compounds applied will reach the membranes of the various fibers at different periods of time. It is easy to see why this type of experimental material is unfavorable. The effect of the inhibitor should be rather fast because a prolonged exposure frequently induces secondary changes which may be irreversible even if the enzyme is reactivated. It would be desirable to have greater leeway between the onset of irreversible inhibition and severe secondary alterations. Low concentrations of Paraoxon did not produce irreversible inhibition for quite a long period of time. The higher concentrations used left only a very short period between irreversible block and severe secondary damage such as decoloration and rigidity. Other organophosphorus compounds have not been tested; some of them may be much more favorable for the aim of these experiments. Obviously, therefore, investigations with more favorable material and more favorable inhibitors are desirable and are planned. However, in spite of all the improvements possible, the data presented have shown that electrical activity in fibers in which it was virtually irreversibly abolished, may be restored by means of a compound known to reactivate specifically the phosphorylated enzyme.

Another significant result of the observations reported is the increase in spike height and amplitude in the muscle fibers exposed to inhibitors of acetylcholin-

RESULTS AND DISCUSSION

Inhibition of lysis

The effects of fluorouracil on phage growth in the synthetic medium are shown in Fig. 1. It is apparent that phage production, as measured by the loss of turbidity of infected cultures, is inhibited only in the even-numbered strain tested. It was found that the multiplicity of infection used, affected the shape of the lysis curves. In cultures infected with T3, a decreasing multiplicity of infection resulted only

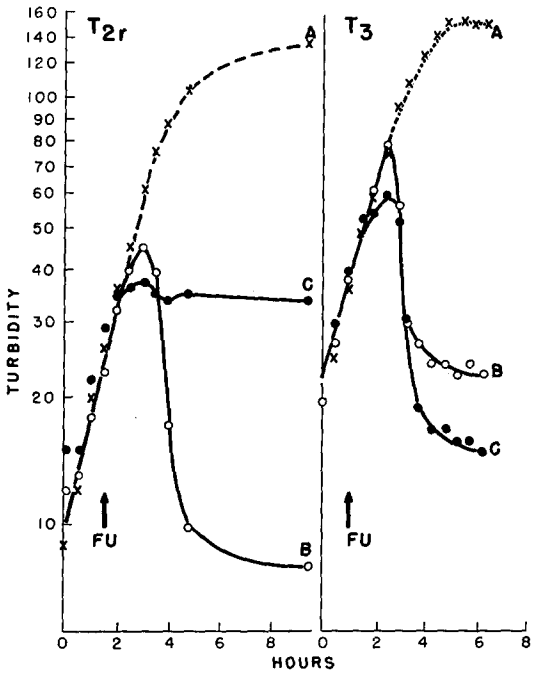

Fig. 1. Effect of 5-fluorouracil on bacterial lysis by phage. FU (50 μg/ml) was added to logarithmically growing cultures of *E. coli* B at the time indicated by arrows. 30 min later, each culture was infected at a ratio of approx. 1 phage particle per 10 bacteria. A, ×—×, control; B, O—O, infected in absence of 5-fluorouracil; C, ●—●, infected in presence of 5-fluorouracil.

TABLE I

EFFECT OF 5-FLUOROURACIL ON MULTIPLICATION OF T2r

In each experiment 50 μg of 5-fluorouracil/ml of logarithmically growing culture of *E. coli* were employed. 0.5 h later, phage were added to the cultures. At completion of lysis, cells and debris were removed by centrifugation and the supernatant fluids were assayed for plaque-forming units.

Phage particles per ml (as multiples of 10⁹)			
	Yield		*Yield ratio 5-fluorouracil/control*
Input	*Control (without 5-fluorouracil)*	*With 5-fluorouracil*	
6	220	1	0.004
60	140	0.7	0.005