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SPONTANEOUS REACTIVATION OF ORGANOPHOSPHORUS-INHIBITED ELECTROPLAX CHOLINESTERASE IN RELATION TO ACETYLCHOLINE-INDUCED DEPOLARIZATION*

W.-D. DETTBARN,†‡ E. BARTELS, F. C. G. HOSKIN§ and F. WELSCH‡

Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, N.Y., U.S.A.

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Abstract—Pretreatment of the electroplax of *Electrophorus electricus* with irreversible inhibitors of cholinesterase (ChE) (diisopropyl phosphorofluoridate, paraoxon or phospholine) increases the sensitivity of the membrane to acetylcholine (ACh). After washing with inhibitor-free Ringer's solution, this effect disappears rapidly. Reapplication of either of the inhibitors restores the initial sensitivity to ACh. Simultaneous measurements of ChE activity show an initial inhibition which is followed by recovery of enzyme activity. The rate of reactivation of the irreversibly inhibited enzyme is highest with paraoxon and lowest with DFP. The return of enzyme activity may be responsible for the loss of ACh action on the membrane of electroplax.

A PARTIAL inhibition of cholinesterase (ChE) is generally assumed to potentiate and prolong the action of acetylcholine (ACh). In fact, this action has been used to explain many of the physiological and pharmacological effects of ChE inhibitors.[1-8] The monocellular electroplax of *Electrophorus electricus* is a suitable preparation for the investigation of the action of ACh on excitable membranes. In the presence of physostigmine, low concentrations of ACh evoke a marked and prolonged maximum depolarization of the membrane. In the absence of an inhibitor of ChE, however, high concentrations of ACh cause a depolarization which is both transient and small in magnitude.[9, 10] Preliminary studies have shown that, after the application of normally irreversible ChE inhibitors, the response to ACh was initially high but decreased with its subsequent application. These findings suggested that the inhibition of the enzyme *in vivo* was not as irreversible as had been shown *in vitro*. Such a reversibility has indeed been reported by several investigators.[11]

The present studies are undertaken to determine whether the decreasing sensitivity of the membrane to ACh could be explained by a partial recovery of the enzyme. To test this, simultaneous measurements of enzymic and electrical activity of the electroplax were made before and after the application of organophosphates. The presence of acetylcholinesterase (AChE) in the electroplax has been demonstrated previously.[12,13]

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† Recipient of a Career Development Award, 5K3NB2513303.

‡ Present address: Department of Pharmacology, Vanderbilt University Medical School, Nashville, Tenn.

§ Present address: Department of Biology, Illinois Institute of Technology, Chicago, Ill.

METHODS

Single electroplax from the organ of Sachs of *Electrophorus electricus* were prepared according to the technique of Schoffeniels.[14] The resting and action potentials were recorded as described previously.[10] The ChE inhibitors used were: *O,O*-diethyl S-(2-trimethyl ethylammonium) phosphorothioate methiodide (phospholine, 217MI); diethyl *p*-nitrophenyl phosphate (paraoxon); diisopropyl phosphorofluoridate (DFP). These compounds were dissolved in eel–Ringer's solution of the following millimolar composition: NaCl, 160; KCl, 5; $MgCl_2$, 2; $CaCl_2$, 2; $NaHP_2O_4$, 3; Na_2HPO_4, 1·2; glucose, 10. The pH was adjusted to 7·0 and the temperature was maintained at 22–25°.

Enzyme activities were determined manometrically and by the colorimetric technique of Hestrin.[15] ACh (5×10^{-3}M) and acetyl-*dl*-*β*-methylcholine (MeCh; 1×10^{-2}M) were used as substrates. Single cells were preincubated with inhibitor for 30 min, washed for varying periods of time with eel–Ringer's solution and then used for enzyme assays. Electrical recordings were made on isolated cells under similar conditions. The enzyme activities of cells not preincubated with inhibitors were used as controls.

RESULTS

In the presence of physostigmine, a reversible cholinesterase inhibitor, ACh reversibly depolarizes the *Electrophorus* electroplax.[10] Both the time course and the magnitude of the changes in the resting potential depend on the concentration of ACh. Thus, each increase in the concentration of ACh is followed by a rapid drop of the resting potential to a new steady state potential. These effects of ACh, however, are not seen in the absence of physostigmine.[10] The effects of pretreatment of an electroplax with DFP, a strong irreversible cholinesterase inhibitor *in vitro*, are shown in Fig. 1. A concentration of DFP several times higher than that required to inhibit ChE

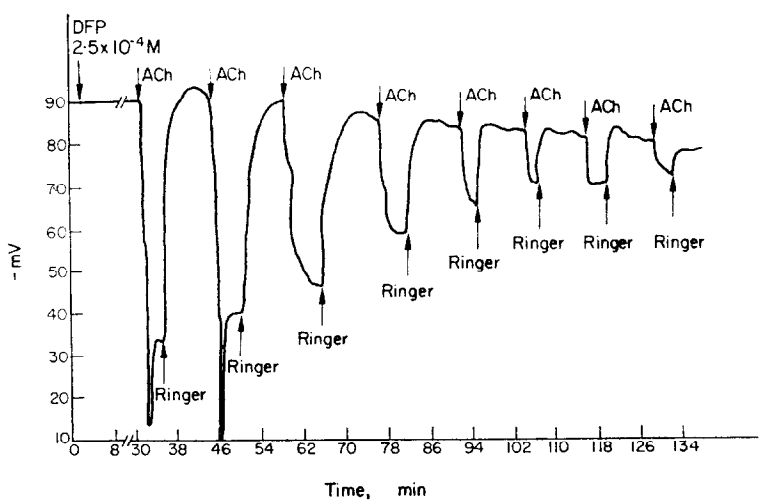

FIG. 1. Decreasing ACh depolarizations after preincubation with DFP. After the cell was incubated with DFP, $2·5 \times 10^{-4}$M, for 30 min, repeated applications of ACh, 5×10^{-6}M, caused decreased depolarizations. The first two depolarizations are 2-phasic.

completely in solution had no effect on the resting potential or, although not shown in Fig. 1, on the pre- or post-synaptic action potential. After removal of the DFP solution and brief washing in eel–Ringer's solution, however, ACh at 5×10^{-6}M caused a rapid depolarization. On return to Ringer's solution, the membrane repolarizes to its original potential. Subsequent application of the same concentration of ACh resulted in a decreasing depolarization of the membrane. It was only after the reapplication of DFP that the original degree of depolarization was evoked. Similar effects of ACh were obtained after pretreatment of electroplax with paraoxon or with phospholine (Figs. 2 and 3). Again, with each subsequent application of ACh, less depolarization was observed. Moreover, after repeated ACh treatment when depolarizations have almost disappeared, a reapplication of the inhibitor restored fully the

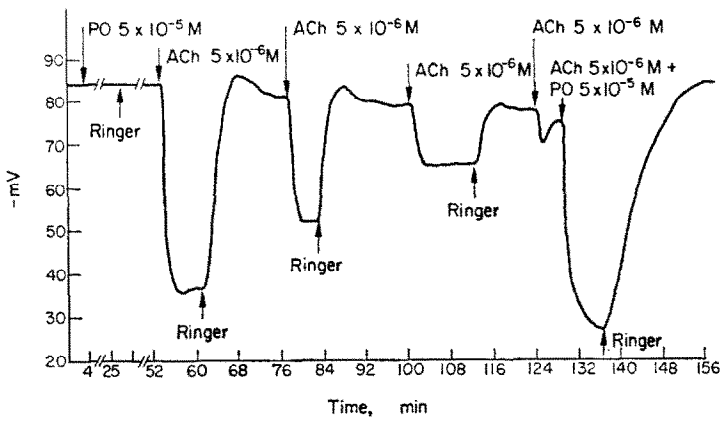

FIG. 2. Reversible action of paraoxon. The cell was preincubated with paraoxon, 5×10^{-5}M, for 30 min then washed in Ringer's solution for 20 min. The depolarization caused by ACh decreased with each application. Adding paraoxon to the test solution at the end of the experiment potentiated ACh again to its maximum response.

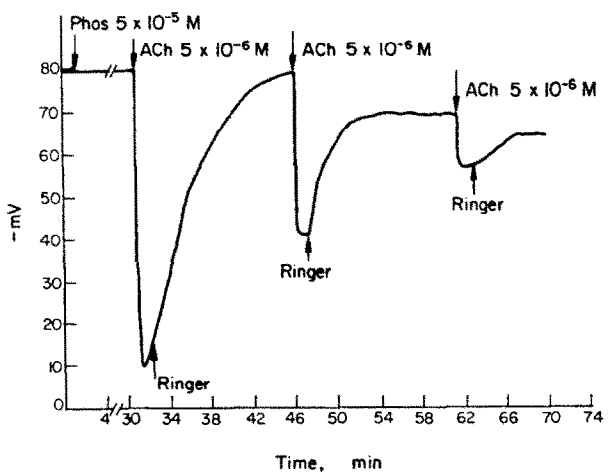

FIG. 3. Reversible action of phospholine. The initial maximum response of the cell to ACh is reduced with each subsequent application.

sensitivity of the preparation (Fig. 2). Figure 4 shows the rate of recovery of ChE activity in intact *Electrophorus* electroplax after treatment with DFP, paraoxon or phospholine. A "least squares" straight line has been arbitrarily fitted to the data, although this may not reflect the actual time course of the reactivation. These three inhibitors are generally considered to be of the "irreversible" type and, since ChE inhibition would be expected to be complete after exposure of a solution of enzyme to these inhibitors at these concentrations (10^{-3} to $2{\cdot}5 \times 10^{-3}$M), the data in Fig. 4 show that a considerable degree of reversibility was measured over a period of time comparable to that of the electrophysiological experiments. It is noteworthy that with

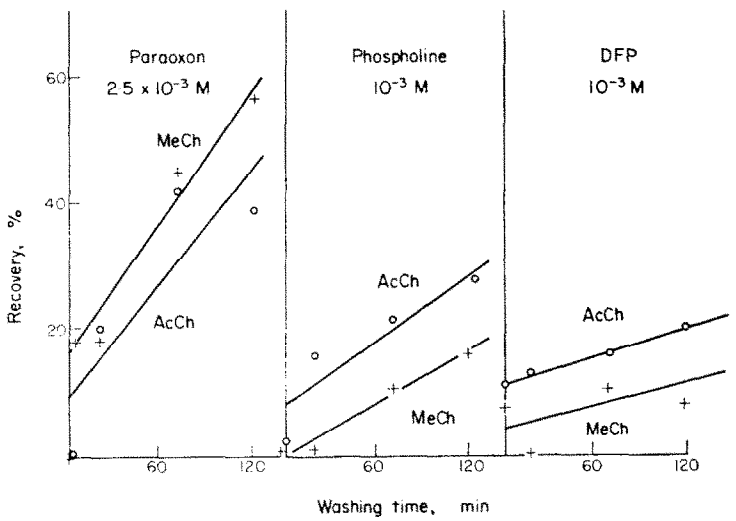

FIG. 4. Time course of recovery of AChE and ChE activity of isolated electroplax after inhibition with paraoxon, phospholine or DFP.

increasing concentrations of inhibitor longer periods of washing were required for complete recovery (Tables 1 and 2). However, the lowest concentration of inhibitor used in our experiments was still very much in excess of that needed for complete inhibition of the enzyme. Uninhibited cells washed for similar periods of time did not show any change in enzyme activity, thus excluding the possibility that a change in the permeability of the cells due to washing could cause the apparent reactivation.

DISCUSSION

When electroplax of *Electrophorus electricus* were treated with organophosphorus ChE inhibitors, which are generally considered to be irreversible, the ChE appeared to recover its activity after removal of the excess inhibitor and washing. Cells treated with DFP showed a slower rate of recovery than did those treated with paraoxon, while the effect of phospholine was intermediate. Although the degree of recovery seemed to depend on whether ACh or MeCh was the substrate, the rate of recovery was approximately the same regardless of the substrate used with a particular inhibitor. The results of the present studies are consistent with those obtained with the purified enzyme; that is, DFP-treated cells would be expected to recover more slowly[16-18] and paraoxon- and

TABLE 1. REACTIVATION OF INHIBITED ELECTROPLAX AChE BY WASHING*

Inhibitor concn (M)	Enzyme activity expressed as % control activity Washing period			
	5 min	30 min	60 min	24 hr
Phospholine				
1×10^{-2}	0	2 (0–3)	6 (0–12)	38 (31–48)
5×10^{-5}		24 (23–25)	30 (20–40)	45 (43–50)
Paraoxon				
$2{\cdot}5 \times 10^{-3}$	0	10 (5–12)	18 (16–30)	
1×10^{-5}	0	18 (10–26)	62 (18–62)	
DFP				
5×10^{-3}	0	0	0	0
5×10^{-4}	0	3 (0–7)	11 (6–15)	
5×10^{-5}	0	7 (3–9)	14 (9–17)	

* Intact, isolated electroplax were washed for varying periods of time after inhibition with irreversible inhibitors of ChE. At the end of each period AChE activity was determined with the Hestrin technique.[15] Number of experiments for each washing period is five. The numbers in parentheses indicate range of values. Mecholyl (1×10^{-2}M) was used as substrate.

TABLE 2. REACTIVATION OF INHIBITED ELECTROPLAX ChE BY WASHING*

Inhibitor concn (M)	Enzyme activity expressed as % control activity Washing period			
	5 min	30 min	60 min	24 hr
Phospholine				
1×10^{-2}	0	7 (7–8)	22 (14–33)	58 (29–100)
5×10^{-5}	4 (0–7)	28 (15–42)	56 (36–80)	62 (58–62)
Paraoxon				
$2{\cdot}5 \times 10^{-3}$	0	11 (5–21)	40 (30–65)	60 (40–91)
1×10^{-5}	8 (4–12)	40 (33–48)	52 (39–62)	73 (72–74)
DFP				
5×10^{-3}	0	0	6 (4–9)	
5×10^{-4}	3 (0–5)	7 (3–9)	30 (18–46)	

* Conditions similar to those described in Table 1. Acetylcholine (5×10^{-3}M) was used as substrate.

phospholine-treated cells more rapidly. Certain variables, however, must be considered which do not readily lend themselves to rigorous control. Paraoxon and phospholine are more potent ChE inhibitors than DFP by approximately an order of magnitude.[19,20]

It is thus noteworthy that both paraoxon and DFP are susceptible to enzymatic hydrolysis,[21] whereas phospholine is probably not inactivated in this manner.[22] Moreover, the possibility that the detoxifying enzymes may be present in lower concentration in *Electrophorus* tissue than in squid axons may not be an overriding

consideration, since these enzymes may be strategically located with respect to the membrane-localized ChE. Paraoxon and DFP are both lipid-soluble and thus should readily cross cellular barriers[23] whereas phospholine, a quaternary ammonium salt, should not penetrate lipid membranes. Although these considerations are not inconsistent with the results of the present studies, they may partially explain the apparent differences in the degree and rate of recovery observed with different inhibitors.

Concerning the functional observations, the time-dependent decrease in the effect of ACh on the membrane potential after pretreatment with organophosphorus ChE inhibitors may be due to various causes, among which are: reactivation of the phosphorylated enzyme; rapid reactivation of nonspecific esterase; *de novo* synthesis of ChE; desensitization of the ACh receptor to ACh; increased permeability of the electroplax due to prolonged washing, making uninhibited enzyme available to ACh. The present studies suggest that reactivation is a reasonable explanation for the functional observations. Whether AChE or nonspecific esterases (ChE) are involved[12] is probably of little consequence, as similar rates of reactivation were observed with a particular inhibitor whether ACh or MeCh was used as the substrate. Although it is known that the rates of spontaneous reactivation or reactivation by nucleophilic agents such as pyridine-2-aldoxime methiodide (PAM) are most rapid for dimethylphosphoryl enzyme, followed by diethyl and diisopropyl, the rates of recovery of the functional parameter (Figs. 1–3) may not follow this order, since this may be an interaction of many factors and components.

It seems unlikely that the rapid recovery of enzyme activity in electroplax can be explained solely by *de novo* synthesis, in view of the low metabolic rate of *Electrophorus* tissue.[24] However, a generally higher labeling of protein in rat brain and lobster nerve after irreversible ChE inhibition has been observed.[25a, 25] In the more actively metabolizing tissues, brain and erythrocyte, spontaneous recovery rates for organophosphorus-inhibited ChE are slower[26–29] and appear to be closely related to the rate of *de novo* red cell formation. More recently the existence of an isozyme of ChE with an unusually short half-life of 3 hr has been described in rat brain.[30] Whether this is valid for the electroplax remains to be shown. The fact that reapplication of the inhibitor restored the sensitivity of electroplax to ACh, as shown in Fig. 2, rules out desensitization. This had been suggested as a possible explanation at the neuromuscular junction,[31, 32] where prolonged exposure of the synaptic membrane to a depolarizing agent reduces the sensitivity to such agents. An increased permeability due to washing has not been observed in untreated cells washed for varying periods of time. Electroplax have also been tested for the presence of detoxifying enzymes which might render the organophosphorus inhibitors inactive. Such enzymatic activity has not been found in this tissue.* Thus the decreasing sensitivity of electroplax to ACh after treatment with irreversible ChE inhibitors appears to result from a recovery of ChE activity.

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* D. Satchel and F. C. G. Hoskin, unpublished observations.

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