microsomal enzymes of guinea pigs do not hydroxylate diazepam.[2] The anticonvulsant activity of diazepam in guinea pigs is therefore mostly correlated in the brain with the presence of diazepam and *N*-demethyldiazepam. In comparison to other animal species guinea pigs are different from either rats or mice in metabolizing diazepam *in vivo*. In fact guinea pigs are similar to mice in respect to accumulation of *N*-demethyldiazepam, but similar to rats for the lack of accumulation of oxazepam.

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Acetylcholine—A possible mechanism for the depolarization response in giant axons of the lobster circumesophageal connective*

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IT HAS been reported that the giant axons of the lobster circumesophageal connective respond to the external application of acetylcholine (ACh) with a membrane depolarization.[1,2] This phenomenon has been of particular interest in view of a proposed role for acetylcholine in axonal conduction.[3] The studies cited have indicated at least two categories of response to ACh in this population of axons: (1) depolarization resulting in an immediate decrease in excitability and spike decrement; and (2) depolarization which initiates spontaneous discharge. It has also been shown that cholinesterase inhibitors, depending on their concentration, fully or partially block these actions of ACh. In the present study we have further examined the relationship between cholinesterase activity and the ACh depolarization in an attempt to clarify a possible interaction and determine whether it reflects a functional role of acetylcholine in the conducted response.

The ligated desheathed connective was mounted in a perfusion chamber[4,5] that permits continuous flow of the bathing medium (2–4 ml/min) with a simple "nontraumatic" switching of solutions. The resting potential and action potentials were monitored via an intracellular glass microelectrode (3–10 megohms) and a conventional high input impedance preamplifier (Bioelectric NF-1). The output was displayed on an oscilloscope and the resting potential was recorded continuously on a strip chart recorder (Varian G-1000) connected to its cathode follower output. The nerve bundle was stimulated electrically with a bipolar electrode driven by a conventional stimulator and isolation unit (Grass). Artificial sea water[1] was the standard perfusion medium. Test substances were dissolved in it and the pH was adjusted to 7·7.

In the population of 7–10 larger giant axons of the circumesophageal connective, we have observed three basic responses to the application of acetylcholine (1×10^{-2} M). Some axons are insensitive even to this high concentration of ACh or respond with a slow depolarization only after sustained exposure (30–60 min). Others begin to depolarize at a moderate rate after 5–10 min of exposure with

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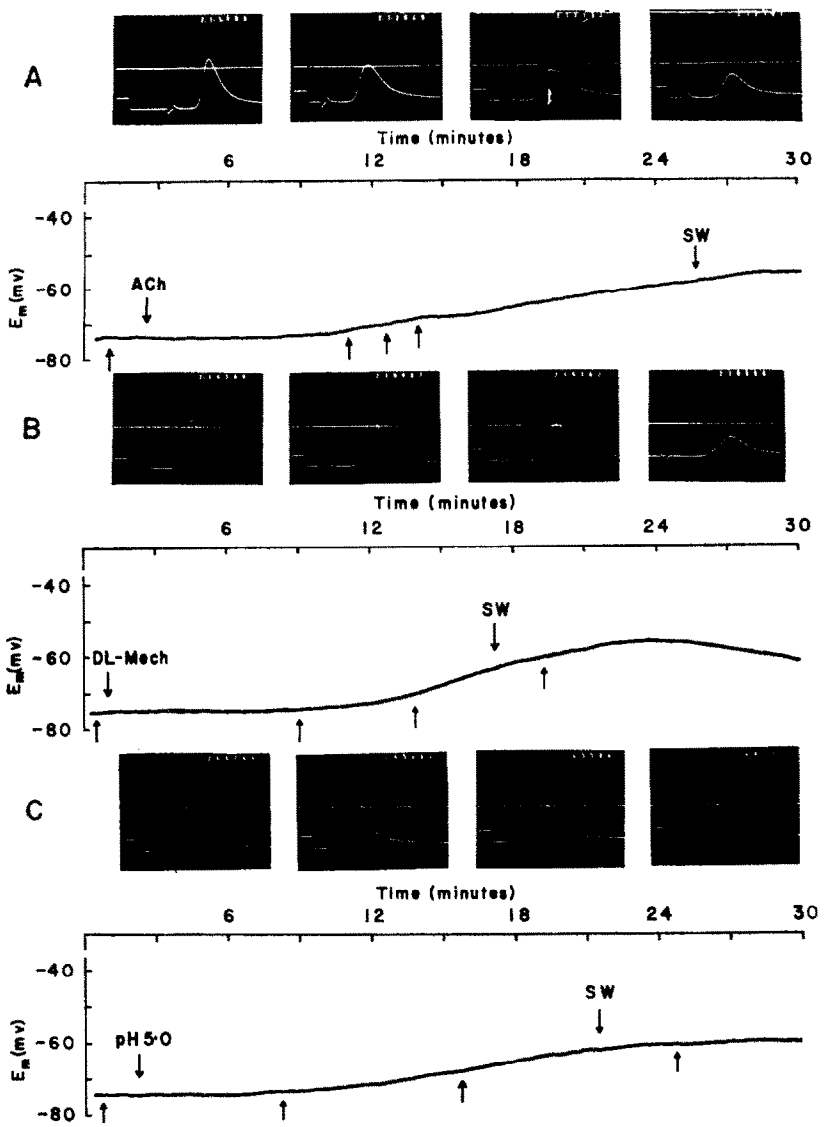

FIG. 1. Strip chart recordings of resting potential showing depolarization and photographic records of changes in the action potential. Calibration pulse is 20 mV, 0·5 msec. The time of each photograph is indicated by markers below the strip chart tracing. (A) Depolarization and spike decrement in response to ACh, 1×10^{-2} M. (B) Depolarization and spike decrement in response to DL-mecholyl (Mech), 2×10^{-2} M. (C) Depolarization and spike decrement in the presence of artificial sea water acidified to pH 5·0 with acetic acid. The marker SW indicates the beginning of washout with normal (pH 7·7) artificial sea water.

conduction block occurring in 10–30 min (Fig. 1a). A third class of response is a rapid depolarization beginning 3–6 min after the introduction of ACh which initiates a spontaneous repetitive discharge This pattern of firing continues for about 5–10 min, retarding the depolarization of the membrane potential, but then depolarization continues with a spike decrement and conduction block, as in the second type of response.

Competitive inhibitors of acetylcholine receptors (atropine and tubocurarine) have been used to attempt a characterization of these responses,[1] but they are either ineffective or else produce alterations of the membrane characteristics that interfere with ACh action. To further characterize the depolarization response, acetylcholine analogues with demonstrated activity at the neuromuscular junction and presumably at the ACh receptor, were tested on the preparation. Carbamylcholine, decamethonium, succinyl choline, and butyryl choline, compounds which either are not hydrolyzed or are hydrolyzed only very slowly by acetylcholinesterase (AChE), had no effect. Acetyl-β-methylcholine (mecholyl) was the only compound other than ACh which elicited depolarization, and the membrane response characteristics were nearly identical to those caused by ACh (Fig. 1b). Most of the tests with this compound were made with a DL-mixture, but a few experiments were performed using the purified D(−)-isomer. It has been reported[6] that only dextrorotatory acetyl-β-methylcholine

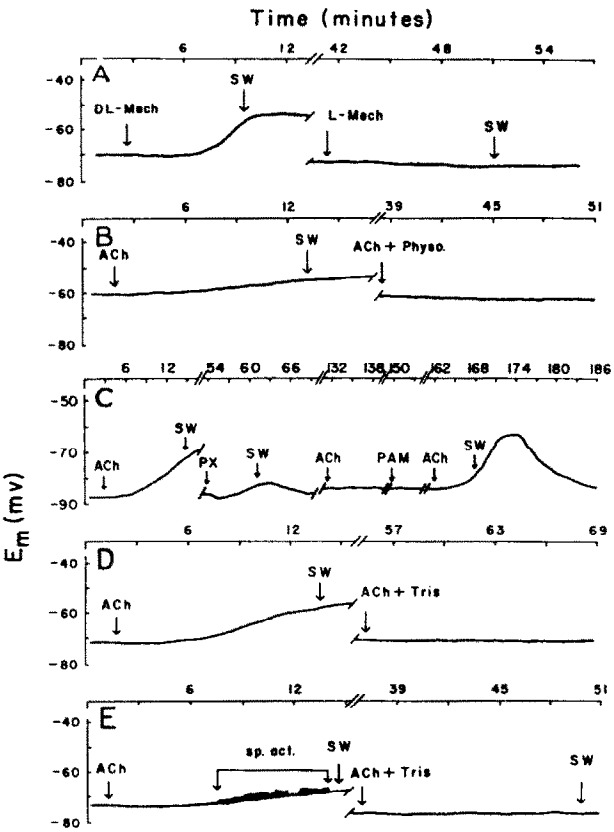

FIG. 2. Strip chart records of the resting membrane potential of lobster giant axons. (A) Perfusion with a DL-mixture of acetyl-β-methyl choline (DL-Mech), $2{\cdot}5 \times 10^{-2}$ M, elicits a depolarization and spike decrement similar to those seen with ACh. D(−)-mecholyl (L-Mech), $2{\cdot}5 \times 10^{-2}$ M, which is not hydrolyzed, is ineffective. (B) Perfusion with 1×10^{-2} M ACh leads to depolarization and spike decrement. Washing with artificial sea water (SW) restores the resting potential and conduction. Perfusion with ACh (1×10^{-2} M) in the presence of physostigmine (Physo), 1×10^{-2} M, had no effect. (C) Control perfusion with ACh (1×10^{-2} M) produces the usual depolarization and spike decrement. Treatment with paraoxon (PX), $2{\cdot}5 \times 10^{-3}$ M, produced a transient effect upon the membrane potential, but after recovery the axon was insensitive to ACh. Treatment with the paraoxon antidote 2-PAM (PAM) for a few minutes restored axonal sensitivity to ACh (1×10^{-2} M). (D) A control perfusion of ACh (1×10^{-2} M) elicits depolarization and spike decrement, while the same concentration is ineffective in the presence of 0·1 M tris. (E) ACh, 1×10^{-2} M, produces depolarization leading to spontaneous activity (sp. act.). Subsequent application of 1×10^{-2} M ACh in the presence of 0·1 M tris is ineffective.

[L(+)] as determined optically is hydrolyzed by the AChE and in our experiments only the mixture containing the L(+)-isomer was effective in producing depolarization (Fig. 2a). To avoid confusion, it should be noted that the dextrorotatory isomer of mecholyl is actually L in terms of absolute configuration.[7] Experiments using inhibitors of nonspecific cholinesterase activity and specific acetylcholinesterase substrates indicate that more than 90 per cent of the activity in lobster axons is due to AChE. Thus it appears that only compounds which are hydrolyzed by the AChE have depolarizing activity and the obvious question arises as to the mechanism of action: Is ACh interacting in a direct fashion with a receptor topographically similar to or integral with the AChE, or is this a secondary effect of the hydrolysis products, choline or acetic acid or both? We have investigated this latter possibility in some detail.

Cholinesterase inhibitors quite specifically block the depolarization response of lobster axons to ACh. Our experiments demonstrate that physostigmine, a so-called reversible inhibitor of cholinesterase activity, completely blocked the ACh depolarization when tested in the concentration range between 1×10^{-2} and 1×10^{-4} M (Fig. 2b). Inhibition of the enzyme is complete even at the lowest of these concentrations. Likewise paraoxon, an irreversible inhibitor, completely blocked the response at concentrations of $2{\cdot}5 \times 10^{-3}$ to 2×10^{-5} M (Fig. 2c), levels which inhibit AChE activity completely. Concentrations of phospholine as low as 1×10^{-6} M were effective in blocking or reversing the depolarization. Treatment with pyridine-2-aldoxime-methiodide (2-PAM) after paraoxon inhibition restored the sensitivity to ACh. Paraoxon as well as other alkylphosphates inactivates the enzyme by phosphorylating the esteratic site of the AChE. This reaction is only slowly or not at all reversible *in vitro*. Experiments with the axons used here indicated that even after 24 hr of washing the enzyme remained inhibited, unless a nucleophilic reactivator was used. One of the most effective reactivating agents is 2-PAM. The effect of 2-PAM in restoring the ACh response makes the possibility of an inhibitor interaction with a receptor topographically similar to ChE seem unlikely.

Perfusion of the preparation with choline bromide in concentrations as high as 2×10^{-2} M had no effect on the resting or action potentials. The local pH change, due to acetate production within or very near the axonal membrane by the hydrolysis of ACh, cannot be calculated with accuracy. However, depolarization and spike decrement similar to those seen in the presence of ACh were elicited by acidifying the perfusion medium to pH 4·0–5·0 (Fig. 1c). Unfortunately, with this procedure the depolarization was usually not completely reversible.

An effect of reduced pH upon the resting potential and conducted response has already been reported[8,9] and is most likely explained by a rather generalized action on the membrane. If the ACh effect is directly related to the increase in local concentration of acetic acid as a hydrolysis product, the presence of a high concentration of a suitable buffer should reduce or block the depolarization. This was seen to be the case for the slow, decrementing spike depolarization (Fig. 2d) as well as for the repetitive firing response (Fig. 2e) when ACh (1×10^{-2} M) was perfused in a bathing solution buffered with 0·1 M tris-hydroxymethylaminomethane (tris). Equivalent results have been obtained using 0·1 M glycylglycine buffer. When washed for 15–30 min in normal bathing solution, the characteristic response to ACh was restored.

The findings reported here support the suggestion that the depolarization of lobster giant axons by ACh is due to localized acidification of the membrane caused by the hydrolyzing activity of AChE.[1,2] The splitting of ACh generates hydrogen ions within or close to the membrane, since the major activity of the enzyme is limited to the axolemma.[10] Thus the shift in pH may cause the observed electrophysiological changes. This is also demonstrated by the antagonistic action of cholinesterase inhibitors such as physostigmine, paraoxon and phospholine on the ACh action.

Bartels and Nachmansohn[11] have shown that organophosphates antagonize ACh reversibly at the receptor site in the eel electroplax preparation when perfused continuously at high concentrations. In our experiments, perfusion with concentrations of inhibitor which assured complete inactivation of axonal AChE were followed by an ACh perfusion only after washing for 15–30 min with normal sea water. As a consequence, no significant levels of unbound organophosphate were present in the perfusion medium, reducing the likelihood that a receptor inhibition of this nature could occur. In electroplax, perfusion of organophosphates at minimum levels, which completely inhibit the ChE, potentiates the response to ACh. In contrast, our studies with lobster giant axon have shown that paraoxon and phospholine are effective in blocking and reversing the ACh depolarization at concentrations as low as 1×10^{-6} M, a concentration which completely inhibits AChE activity. Concentrations that partially inhibit the AChE activity reduce the magnitude of the ACh depolarization in proportion to the percentage of inhibition.[2] Several other reversible and irreversible ChE inhibitors have been shown to prevent or reverse the ACh depolarization.[1,2] In our experiments with these axons, using a variety of ChE inhibitors in a broad range of concentrations (1×10^{-2} to 1×10^{-6} M), no potentiating effect on the ACh action was ever observed. This is in contrast to the potentiation by ChE inhibitors of the ACh action at synaptic or junctional sites. Any interpretation

of the effects of organophosphates at high concentrations must take into account possible interactions with other enzymes and proteins of the membrane.[2, 12] It seems likely that such interactions may be responsible for the transient conduction block after treatment with high concentrations of inhibitor, since there is no detectable ChE activity. Recent experiments on squid giant axon showed a reversible attenuation of the spike in the presence of the organophosphate ChE inhibitor, Tetriso, at 5×10^{-3} M, a concentration 50,000 times that necessary for complete inhibition of ChE activity.[13] In our experiments with lobster axons, the effects of irreversible inhibitors on the ACh depolarization could be obtained with inhibitor concentrations which produced no effect by themselves on the resting potential or the conducted response.

The experiments demonstrating the lack of action when the non-hydrolyzable isomer, D(−)-mecholyl, or other nonhydrolyzable cholinergic compounds such as carbachol and decamethonium were used support the role of AChE in causing the effect of ACh on the membrane and action potential of the lobster giant axons. In this regard it should be noted that the best effects of ACh could be observed only at a concentration of 1×10^{-2} M, which corresponds with the optimal substrate concentration for AChE activity in intact lobster preparations; lower ACh concentrations (1×10^{-3} M) are much slower in causing the action described above.

A very similar type of response has been described for eel electroplax[14] in which the receptor sites have been inactivated by hypertonic solutions, leaving the ChE activity unaltered. These preparations are no longer sensitive to lower concentrations of ACh and have no sensitivity at all to nonhydrolyzable analogues. A depolarization of the residual resting potential was produced only by concentrations of ACh or mecholyl greater than 10^{-3} M, and this depolarization was blocked by physostigmine, difluorophosphate, or the presence of strong buffers. These observations correspond with those reported here for lobster giant axon, such as the levels of ACh necessary for depolarization, the effect of ChE inhibitors in blocking depolarization, the efficacy of well-buffered bathing solutions in blocking ACh action, and the fact that part of the ACh effect can be mimicked by changes in the pH of the bathing solution. It was suggested that for the specially treated electroplax preparation the action of ACh was dependent on the hydrolyzing activity of the ChE, and it would appear likely that this is the case in lobster giant axon as well. On the basis of these experiments, it appears that the ACh depolarization responses described here are dependent upon cholinesterase activity, in particular the accumulation of acetic acid as a hydrolysis product.

It is possible that the mechanism is similar to that demonstrated recently by Walker and Brown[15] for certain cells of Aplysia ganglion. Decreases in the extracellular pH were shown to increase the membrane conductance to chloride. Hyperpolarization, depolarization, or no response was elicited, depending upon the value of the chloride equilibrium potential relative to the resting potential for the cell. The changes in resting potential and the conducted response of lobster axons may very well be related to such a change in conductance, although it is not possible on the basis of the available data to designate the ion involved.

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