AN IMPROVED ISOLATED SINGLE ELECTROPLAX PREPARATION

I. EFFECT OF COMPOUNDS ACTING PRIMARILY AT THE SYNAPSES

PHILIP ROSENBERG, HENRY HIGMAN AND DAVID NACHMANSOHN

Departments of Neurology and Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

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SUMMARY

Acetylcholine and related structures have been tested as to their effects on the electrical activity of an improved isolated single electroplax preparation. As a measure for comparing the relative strength of the compounds, the concentration which produced 50 % decrease in spike height within 20 min or less (usually from 5–15 min) was used. This concentration is referred to as the minimum active concentration. (a) Acetylcholine, decamethonium and *d*-tubocurarine were about 5–10 times as active as in previous preparations; no marked increase in sensitivity was observed with neostigmine and dimethylaminoethyl acetate. (b) Among the newly tested compounds succinylcholine proved to be twice as strong as acetylcholine, propionylcholine about 100 times weaker, and benzoylcholine several hundred fold weaker; the S-analogue of acetylcholine was about 3–5 times weaker. Dimethyl curare is only about half as strong as curare. (c) The decrease in spike height caused by the various quaternary compounds tested showed much better reversibility than in the previous preparation. (d) Curare in concentrations about 1000 times as high as those required to block the indirect response, abolished the propagated spike evoked by direct stimulation. In previous preparations no effect was obtained with curare outside the synaptic region even in higher concentrations than those applied in the present studies. (e) Following block of the indirect spike by curare, acetylcholine when applied in higher concentrations was able to overcome the block thus adding new evidence for the competitive action between acetylcholine and curare for the receptor. Dimethylaminoethyl acetate and the S-analogue of acetylcholine did not affect the direct spike in presence of curare in the concentrations tested. (f) Dimethylaminoethyl acetate acts much more strongly at pH 6 to 8, where it is present mostly in the cationic (protonated) form, than at pH 9, where it is present mostly in its uncharged form. The role of the Coulombic forces in the receptor protein thus appears similar to that previously observed with acetylcholinesterase. (g) The various changes of the response to acetylcholine and related structures after the removal of the adjacent membrane are a new demonstration that the effect of externally applied compounds on biological membranes depends to a great extent on the permeability barriers protecting the active membrane.

Abbreviations: DMAEA, dimethyl aminoethyl acetate; ACh, acetyl choline; S-ACH, acetoxyethyl dimethyl sulfonium sulfate; TBC, tubocurarine chloride.

INTRODUCTION

Electric organs of electric fish have been used, since 1937, by NACHMANSOHN and his colleagues for elucidating the role of acetylcholine in the generation of bioelectric currents. The results up to date have been summarized in a recent monograph[1]. During the last decade isolated rows of electroplax of the electric eel (*Electrophorus electricus*) have been used in the laboratories of CHAGAS AND NACHMANSOHN for the analysis of electrical characteristics and their relation to the effects of acetylcholine and related compounds known to react with the acetylcholine system (see for instance ref. 2–6). However, due to the large number of cells in these preparations and the presence of many extracellular membranes surrounding the electroplax, forming strong permeability barriers for many compounds, the results were in many respects unsatisfactory. In particular, they were completely unsuitable for any investigation of ion movements associated with electrical activity and the study of chemical effects on such movements.

A new important development started with the preparation of a single isolated electroplax by SCHOFFENIELS during the years 1956 to 1958 (see ref. 7–9). A single cell is mounted in a chamber in such a way that it separates two pools of fluid, one pool bathing the innervated conducting membrane, the other the noninnervated and nonconducting membrane. Thus, both membranes can be exposed separately to chemical compounds and the effects of the exposure on ion flux and electrical activity can be examined. In the first observations of SCHOFFENIELS in which the effects of acetylcholine and related structures were studied, an extracellular membrane of the adjacent compartment was still attached to the innervated membrane[7]. Later SCHOFFENIELS improved this preparation by removing this membrane through dissection. Only a minimum amount of ground substance at most 100 μ thick but possibly less, is still present in front of the conducting membrane of the cell. SCHOFFENIELS initiated studies on ion flux with this improved preparation[8,9] and these studies were later continued in this laboratory by WHITTAM AND GUINNEBAULT[10,11].

It appeared necessary for many reasons to re-xamine and re-evaluate systematically the effects of acetylcholine and related structures with the improved preparation of SCHOFFENIELS. Such study promised to provide pertinent quantitative data as to the effects of the compounds on the innervated membrane without interference of a strong extracellular structure which must have greatly modified these effects. This and the following paper present the results of these investigations.

The innervated membrane contains a large number of synapses, about 20 to 50,000 per electroplax, dependent on the size of the cell. This paper summarizes the results obtained with compounds acting primarily on the synaptic junctions. The second paper describes effects of compounds acting on both synaptic and conducting membranes.

METHODS

The procedure used for the isolation of a single electroplax was essentially the same as described previously[6–8]. A slice of tissue was cut from the caudal part of the electric eel (organ of SACHS); a single row of cells was isolated from the slice and tied in a frame and covered with oxygenated Ringers' solution. The cells were dissected under microscopic control as close as possible to the innervated membrane.

Only undamaged electroplax were used in the experiments. A single electroplax unit was mounted between two pools of Ringer's solution in a special chamber. The dissection allowed the innervated membrane of the cell to be placed firmly and directly against a window (8 mm long and 0.4 mm wide) punched out of a sheet of nylon. The chamber permits washing of the two cellular membranes by separate pools of oxygenated Ringer's solution. Compounds dissolved in one pool could pass to the other pool only through the cell. All compounds were added to the pool bathing the innervated membrane unless otherwise stated. The compounds were dissolved in Ringer's solution of the following composition in μmoles/ml: NaCl, 160; KCl, 5; $CaCl_2$, 2; Na_2HPO_4, 1.2; NaH_2PO_4, 0.3 and glucose, 10. The pH was 7.0 to 7.2 except where stated when the pH was made more alkaline or more acid by addition of NaOH or HCl.

The electrical activity was recorded with extracellular electrodes dipped in the solutions bathing the innervated and noninnervated membranes. The cell was stimulated by monophasic pulses of 0.1 msec duration and of controlled recorded intensity. The magnitude of the action potential in mV was recorded from a cathode ray oscilloscope. The conducting membrane of the electroplax was stimulated either directly by a cathodic pulse applied to the innervated membrane or indirectly by reversing the polarity of the stimulus[5].

RESULTS

Table I summarizes the compounds tested. In low concentrations they affect electrical activity by acting primarily at the synapses. The concentration required for decreasing the height of the response by 50% within 20 min or less has been taken as criterion for the strength of action of a compound rather than complete block because with some compounds very small potentials may be elicited for a long time. These are probably local, nonpropagated responses and may persist for a long time

TABLE I

MINIMAL ACTIVE CONCENTRATIONS* OF ACETYLCHOLINE AND RELATED STRUCTURES WHICH AFFECT ELECTRICAL ACTIVITY OF THE ISOLATED SINGLE ELECTROPLAX BY ACTING PRIMARILY AT THE SYNAPSES

The first six compounds listed were tested in the presence of 34 mμmoles/ml of physostigmine.

Compounds	*No. of Expts.*	*Minimal active concentration*	
		mμmoles/ml	*Time min*
Acetylcholine	7	2–4	5–10
Dimethylaminoethyl acetate	7	200–1500	5–20
Propionylcholine	3	250–500	8–18
Benzoylcholine	3	600–1200	11–16
Succinylcholine	3	1.5	8–17
β-acetoxy ethyl dimethyl sulfonium sulfate	3	12	5–10
Carbamylcholine	3	25–50	5–20
Decamethonium	4	2–4	5–10
Neostigmine	3	50–100	4–20
d-tubocurarine	6	1–7	6–16
Dimethylcurare	2	3	2–10

* Concentration required to decrease spike height by 50% within 20 min or less (see last column).

after the propagated spike potential has been abolished. With other compounds no such local responses are observed. Therefore, the complete abolition does not appear to be the best measure for comparing the strength of a compound. The difference in block of electrical activity is not surprising. Although all the compounds tested affect electrical activity by reacting with the acetylcholine system, their affinity to the various protein members of the system differs greatly[1]. Therefore, additional factors are introduced when complete block is taken as the measure of the strength of compounds not affecting electrical activity in a strictly identical and comparable manner, *i.e.*, by the reaction with the same protein and with the same relative affinity: in one case local responses may be elicited but not in another. A 50% decrease of the response within a given time has therefore been selected as an indication of comparative strength. The concentration required to produce this effect has been referred to as minimal active concentration. All compounds, with the exception of dimethylcurare, were tested on cells of two or more electric eels.

The cells showed relatively small variations in their response to most of the compounds tested. Surprisingly, however, the response to curare was not regular. The greater part of the cells responded to a few μg/ml of curare (see Table I) but some of them were resistant to concentrations as high as 100 μg/ml. These curare resistant cells responded nevertheless to other quaternary compounds, such as carbamylcholine and decamethonium, in the usual concentrations. We do not have, at present, an explanation for this irregularity in behavior towards curare.

According to theory, the acetylcholine system is essential for the generation of bioelectricity[1]. However, acetylcholine and many other closely related quaternary nitrogen compounds, when applied externally, do not affect conducting membranes of nerve and muscle fibers. The compounds are lipid insoluble and their failure to act on axonal conduction has been attributed to the presence of structural barriers preventing the compounds from reaching the active site. This viewpoint has been experimentally supported in various ways[12,13]. However, in the electroplax they abolish electrical activity and simultaneously depolarize the cell. This simultaneous block of the response to both direct and neural stimulation was first assumed to indicate the absence of a structural barrier protecting the conducting membrane of the electroplax of *Electrophorus electricus* against these types of compounds. However, *d*-tubocurarine (curare) blocks in low concentration only the indirect spike. Even in high concentrations the direct spike appeared in earlier experiments to be unaffected. When acetylcholine and other depolarizing agents were applied in presence of low concentrations of curare, the direct spike was not affected, contradicting the assumption of the absence of structural barriers. Since the conducting membrane of an electroplax contains many thousands of synapses, depolarization of so many points of the membrane produces a short circuit of the entire membrane and it is this short circuiting which is responsible for the block of the direct spike and not a direct action[6].

Curare does not depolarize. Therefore, it cannot produce a short circuiting of the conducting membrane. In low concentrations, about 1 mμ mole/ml, it blocks the indirect but not the direct spike. However, if applied in 1 μmole/ml, it has now been observed with the new improved preparation, that it does block the direct spike (Fig. 1). Apparently, when used in high concentrations, small amounts of curare are able to penetrate and these small amounts are sufficient to act directly upon the conducting membrane. The barrier is relative and not absolute.

A small amount of physostigmine was always present in the solutions containing esters which are hydrolyzed by acetylcholinesterase. The concentration of physostigmine added was about 0.1 the minimal amount necessary to affect either the direct or the indirect spike. Acetylcholinesterase is present in several fold excess. Physostigmine in the small concentrations used inhibits most likely a fraction of cholin-

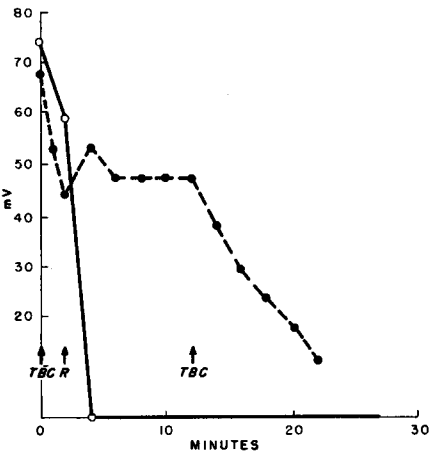

Fig. 1. Effect of TBC in high concentration on the direct response of the isolated single electroplax of *Electrophorus electricus*. The compound was added to the pool bathing the innervated membrane. The response (in mV) to direct ●---● and indirect O—O, stimulation, was recorded by an oscilloscope using extracellular electrodes. In the first exposure 3500 mμmoles/ml of TBC were used, in the second 1400 mμmoles. At R the preparation was washed with Ringer's solution.

esterase, and possibly other esterases, which permits the externally applied ester to reach the active site and to act before it is hydrolyzed. In the absence of physostigmine, about thousand times as much acetylcholine is required for blocking electrical activity, as in its presence. DMAEA, the tertiary analogue of acetylcholine, is also capable of depolarizing the synaptic junction; however, much higher concentrations are required[4].

The potency of DMAEA did not vary significantly when applied at a pH range from 6 to 8. When, however, the pH was adjusted to 9.3, its potency fell drastically to about 15 to 25 % of that observed at pH 6–8 (Fig. 2). Tris(hydroxymethyl)aminomethane in 1.5 m*M* concentration, was used as the buffering agent in these experiments instead of phosphate buffer in order to avoid precipitation at alkaline pH. The buffer did not affect the electroplax action potential.

In the first observations with the isolated single electroplax preparation it was possible to obtain recovery of electrical activity after block by tertiary nitrogen

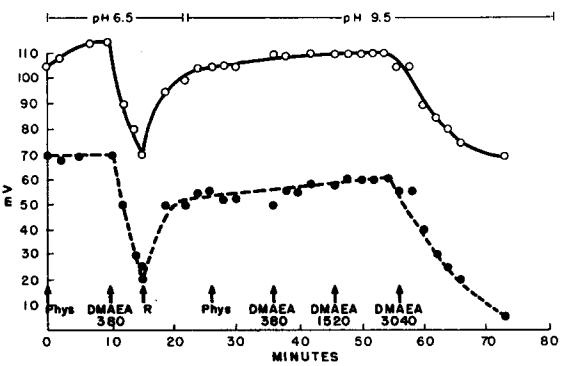

Fig. 2. Effect of DMAEA at pH 6.5 and 9.5 on the electrical activity of the isolated single electroplax. Recordings and symbols as in Fig. 1. Physostigmine (Phys), in a concentration of 34 mμmoles/ml was added 10 min prior to DMAEA and was always present when the preparation was exposed to the ester. The concentration in mμmoles/ml are indicated by the figures below DMAEA.

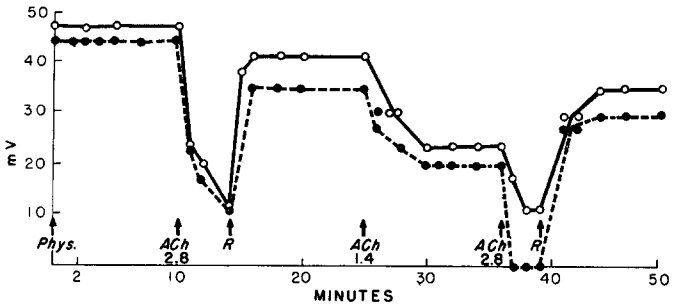

Fig. 3. Reversibility of ACh action on the electrical activity of the isolated single electroplax. Recordings and symbols as in Fig. 1. Physostigmine, in 34 mμmoles/ml concentration was added 10 min prior to ACh and was always present when the preparation was exposed to the ester. The figure below ACh indicates its concentration in mμmoles/ml.

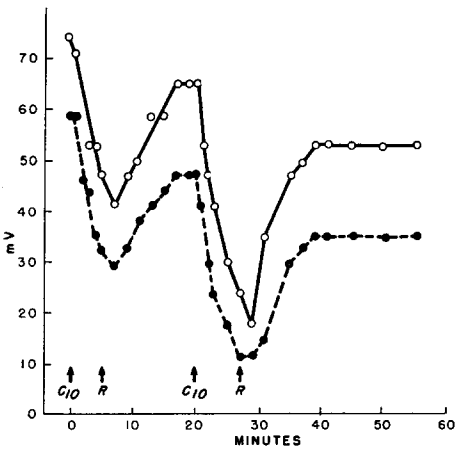

Fig. 4. Reversible effect of decamethonium iodide (C_{10}) on the electrical activity of the isolated single electroplax. Recordings and symbols as in Fig. 1. The concentration of decamethonium iodide applied was 2 mμmoles (0.5 μg)/ml.

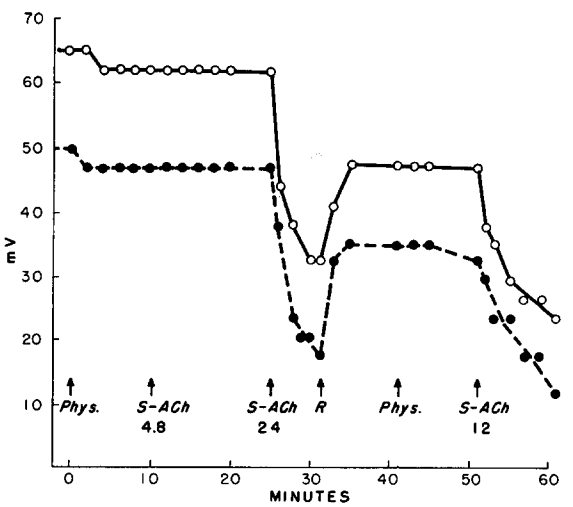

Fig. 5. Effect of B-acetoxyethyl dimethyl sulfonium acetate (S-analogue of ACh) on the electrical activity of the isolated single electroplax. Recordings and symbols as in Fig. 1. Physostigmine, in a concentration of 34 mμmoles/ml was added 10 min prior to S-ACh and always present when the preparation was exposed to the ester. The concentrations in mμmoles/ml are indicated by the figures below S-ACh.

compounds. However, recovery after block by quaternary compounds was only partial and transitory. With the improved preparation the recovery has now been found to be much more marked and lasting. Figs. 3 to 5 show results obtained on testing the reversibility of ACh, decamethonium (C_{10}) and the sulfur analogue of acetylcholine, B acetoxyethyl dimethyl sulfonium sulfate. The mV recorded are not the actual potentials developed by the cell, since external and not intracellular electrodes were used. Whereas previously the block produced by acetylcholine was only reversible to the extent of 0–40 % and sometimes only transitory, we now find a much stronger and sometimes even complete and persisting reversal of block. In the present experiments the inhibition of electrical activity by decamethonium could be at least 50 % reversed, whereas previously no reversibility was obtained with this compound. We also found a marked reversal of the effect of carbamylcholine on the height of the action potentials (50 % or more) and the results with succinyl choline, prostigmine, DMAEA and S-ACh were similar.

It was previously shown that by increasing the concentration of carbamylcholine it was possible to overcome the block by curare[6]. Table II shows results obtained when ACh, DMAEA and S-ACh were applied to the improved preparation following

TABLE II

EFFECT OF ACETYLCHOLINE AND ANALOGUES ON THE DIRECT RESPONSE OF THE ISOLATED SINGLE ELECTROPLAX IN THE PRESENCE OF CURARE

The minimal active concentrations of ACh, DMAEA and the S-analogue of acetylcholine were tested. Curare in the final concentrations listed was applied to the fluid bathing the innervated membrane. The indirect response was blocked by curare prior to the application of ACh, DMAEA and S-ACh. The compounds were tested in the presence of the same concentration of curare and in the presence of 0.034 μmoles/ml physostigmine.

Curare μmcles/ml	*Compound*	*Minimal active concentration μmoles/ml*	*Highest concentration tested μmoles/ml*	*Time min*
0.07	S-ACh	No effect	0.24	
0.07	DMAEA	No effect	30.0	
0.007	ACh	0.06–0.14		8–9
0.07	ACh	0.28–0.56		2
0.35	ACh	5.6		20

curare. About 50 times the minimal active concentration of acetylcholine is required to overcome block at the synapse by 7 mμmoles/ml curare, while 2000 times the minimal active concentration was required to overcome block by 350 mμ moles/ml curare. These findings add new support for the competitive nature of the action of acetylcholine and curare on the receptor.

However, since it has now been shown that curare in high concentrations does affect the conducting membrane outside the synaptic region, it is at present not possible to ascertain whether ACh in the high concentration used overcame the block only at the synapses or acted also directly on the receptors in the membrane conducting the propagated spike.

Fifty times the minimal active concentration of DMAEA at pH 7 or 8 did not overcome curare block; since this concentration in absolute figures is quite high, higher concentrations were not tested.

DISCUSSION

The compounds tested in this paper and that which follows have been divided into two groups according to their effects on the electrical activity of the cell[1,5]. The members of one group, mainly quaternary ammonium derivatives, block electrical activity, by reacting primarily with the receptor at the synapses and simultaneously depolarize the membrane. These compounds are referred to as receptor activators. The members of the second group, referred to as receptor inhibitors, block electrical activity but do not cause depolarization. The tetrahedral structure of the quaternary nitrogen group apparently induces a change of configuration of the protein in which the protein envelops the more or less spherically shaped cationic group. The possibility of such a folding was suggested by the observation that the activities of acetylcholinesterase and choline acetylase are greatly enhanced by the presence of an extra methyl group compared to those when the tertiary analogues are used[14]. Experimental evidence supporting the assumption of a configurational change was offered by the observations on the change of entropy of activation (ΔS^*) by the hydrolysis of acetylcholinesterase: it is very unfavorable in the case of the tertiary analogue of acetylcholine and becomes very favorable in the case of acetylcholine, *i.e.*, in the presence of the extra methyl group[15]. Local changes in configuration of the receptor protein may lead to the removal of strategically located positive charges in the membrane, thereby acting as the trigger for accelerated ion flux.

The compounds discussed in this paper are receptor activators except curare and dimethyl curare, which are receptor inhibitors. The sulfur analogue of acetylcholine has not yet been tested for its ability to produce depolarization, so that it is not yet known whether it is a receptor activator or inhibitor. This compound is similar to acetylcholine in many ways, *i.e.*, it reacts in small concentrations with the receptor, it is ineffective in the usual concentration following curare, and it has a charged group. But it differs in that there are only two methyl groups on the sulfur atom compared to the three on the nitrogen in acetylcholine. It will be interesting to determine whether this compound has a depolarizing action and whether the ΔS^* of its hydrolysis by acetylcholinesterase is favorable, thus indicating a configurational change of the protein.

Previously, when rows of cells were used or single cells not as finely dissected as in the present experiments, the concentration of acetylcholine required for blocking electrical activity was about 30–60 mμmoles/ml or more. Although the measure of effective strength is not the same as that used in previous experiments, the ester acts on the improved preparation in concentrations of about 2–4 mμmoles/ml. Moreover, the reversibility of the action is now much more marked and satisfactory than previously. The method of applying acetylcholine is still extremely crude compared with the biological process, in which acetylcholine is simultaneously liberated in a period of μsec in thousands of synapses within the active membrane. Under those conditions still very much smaller amounts, probably by several orders of magnitude, must be assumed to be sufficient for the generation of bioelectricity. The crude method of applying acetylcholine and possibly the presence of thousands of synapses may make it difficult to activate simultaneously the amount of receptors required for the electrogenic action, so that only a blocking effect is observed. Synchronization may be a pre-requisite for obtaining the postsynaptic potential. More-

over, by recording the overall activity of what is occurring at synapses without synchronization it may be quite difficult to observe any positive electrogenic effect at any one synapse, since very rapidly excitation may be followed by block.

Comparing the concentrations of some of the other compounds with those previously required for block, we find decamethonium and curare to act in 5–10 times lower concentrations than those used previously. Neostigmine and DMAEA, on the other hand, acted in concentrations similar to those reported previously. Most of the other compounds have not been tested before on the electroplax. Succinylcholine is, on a molar basis, twice as potent as acetylcholine. The compound resembles two acetylcholine molecules combined. Diquaternary nitrogen derivatives in which the two quaternary nitrogens are separated by a distance of about 14 to 15 Å, are much stronger bound to acetylcholinesterase[16] and apparently to the receptor protein[17] than corresponding monoquaternary compounds. There is apparently a second negative charge in the surface of the protein at this distance from the anionic site; thereby the binding is greatly enhanced. Since each of the quaternary groups of succinylcholine should be able to produce the folding, the stronger binding by the other nitrogen readily accounts for the stronger action. The specificity of the receptor for acetylcholine appears quite remarkable when one compares the potency of propionylcholine with that of acetylcholine: one additional methyl group in the acyl decreases the potency hundred fold. This may be due to steric hindrance.

The removal of a single methyl group from the quaternary nitrogen of acetylcholine as in DMAEA decreases the potency about 200 fold. Here, however, different factors must be considered: DMAEA, although a tertiary amine, is capable of causing depolarization[5]. DMAEA has a pK of 8.3 and therefore it will exist at pH 6–8 from 99% to 67% in its charged form in which the methyl group of the quaternary nitrogen analogue is replaced by a proton. It appears likely that the charged form is the active one: the substitution apparently greatly weakens but does not abolish completely the activating effect on the receptor protein. This view is supported by the fact that no marked change in the potency of DMAEA was observed when the pH was changed in the range from 6 to 8. In this range the percentage of the charged form was reduced only by about one third. This decrease may be too small to produce a significant effect considering the complexity of biological material. At pH 9.3, however, the potency of DMAEA decreased by a factor of 4–8, corresponding roughly to a decrease in percentage of charged DMAEA to about one sixth of what it is at pH 8. This observation strongly supports the assumption that it is indeed the protonated cationic form of DMAEA which is the active species. The demonstration of the importance of Coulombic forces in the interaction between the small molecule and the receptor protein is of particular interest when compared with the observations on the hydrolysis of ACh and DMAEA by acetylcholinesterase[18]. The rate of hydrolysis of the tertiary analogue is lower than that of the quaternary form, but it does not differ markedly between pH 6 and 8; at pH 9, however, the rate has strongly decreased. It is interesting to note here again, as in other cases, the apparent similarity of molecular forces effective in the active surface of the proteins of the acetylcholine system.

The actual pH in the conducting membrane of the electroplax in these experiments is, of course, not known. Only the external pH of the surrounding fluid has been changed. However, since the potency of DMAEA did change markedly with

the pH of the external fluid, it seems possible that the pH in the conducting membrane, at least at the poorly protected region of the synapse, may have been altered to some extent.

Using the improved preparation, it has been possible for the first time to block the direct response by the action of curare. About a thousand times higher concentration of curare is required to block the response to direct stimulation than that which blocks the response to neural stimulation. This effect serves to emphasize the point that although the conducting membrane is protected by a strong permeability barrier in contrast to the synapses there is no fundamental difference between the membrane and the synaptic areas as far as the primary role of the acetylcholine system is concerned, since curare is able to act on both sites. A strong further support are the recent findings of DETTBARN[19] that curare is capable of blocking rapidly and reversibly electrical activity at the RANVIER node.

The greater sensitivity of the preparation, the better reversibility of the effects and the action of curare on the propagated spike emphasize again the importance of permeability in evaluating effects of compounds on the intact cell.

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