AN IMPROVED ISOLATED SINGLE ELECTROPLAX PREPARATION

II. COMPOUNDS ACTING ON THE CONDUCTING MEMBRANE

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(Received April 23rd, 1960)

SUMMARY

Effects on the electrical activity of an isolated single electroplax preparation were studied with several compounds which act at the conducting membrane as well as at the synapses. Two types of compounds were tested:

1. Tertiary amines. Some of them are potent inhibitors of acetylcholinesterase and of the acetylcholine receptor (physostigmine and the tertiary analogue of Prostigmine). Others are mainly receptor inhibitors: procaine, tetracaine, and dibucaine ("local anesthetics") and atropine. All these compounds block electrical activity without depolarization. Their reaction with the receptor protein has been recently demonstrated in solution.

2. Lipid soluble quaternary ammonium ions: pyridine aldoxime dodeciodide, noracetylcholine, and norcholine. These compounds act on the acetylcholine system, but the affinities to the various protein members seem to be influenced by concentration and structure; depending on a variety of factors block of response may be observed with or without depolarization. Atropine, norACh and norCh affected the indirect response in about 0.1 the concentration required to affect the directly evoked spike. The other compounds affected the response to both types of stimulation about equally. All compounds tested affected the directly evoked spike in approximately the same concentration in the presence as in the absence of curare. Physostigmine and T.P. are twice as potent blockers of electrical activity at pH 6 as at pH 9. At pH 6 the molecules, in solution, are present in their cationic, at pH 9 in their uncharged form.

INTRODUCTION

In a preceding paper[1], using the improved preparation developed by SCHOFFENIELS[2], we have described the effects of acetylcholine and related quaternary nitrogen structures which act primarily at the synaptic regions of the electroplax. In the present paper

Abbreviations: TP, tertiary analogue of prostigmine; PAD, pyridine-2-aldoxime dodecyliodide; ACh, acetylcholine; M.A.C., mininal active concentrations; DIB, dibucaine; AT, atropine; PHYS, physostigmine.

* BT-519.

** BT-526.

we will examine the effects upon electrical activity of compounds which penetrate structural barriers more readily since they are lipid soluble and therefore act upon the acetylcholine system of the conducting as well as of the synaptic membrane of the electroplax. They are mostly tertiary amines which combine with the acetylcholine receptor, but apparently do not readily produce a change of configuration due to the absence of the tetrahedral quaternary nitrogen group. They block the response to direct and indirect stimulation without depolarization. These compounds are, therefore, called receptor inhibitors[3]. Some of them are potent cholinesterase inhibitors. The directly evoked spike is blocked in about the same concentration in the presence of curare as in its absence. To determine the relative potency of the charged *versus* uncharged amine, physostigmine and T.P. were tested at pH 6 and 9.

Some experiments were also performed with lipid soluble quaternary ammonium ions in which a dodecyl group substitutes one methyl group on the nitrogen. The compounds studied are PAD, β-acetoxyethyl dimethyl dodecyl ammonium iodide (noracetylcholine dodeciodide-norACh 12), and β-hydroxyethyldimethyl dodecyl ammonium iodide (norcholine dodeciodide-norCh). These lipid soluble analogues of acetylcholine block the direct response of the cell in the presence of curare like tertiary amines, but in contrast to the latter they are able to depolarize[4].

The methods used for isolating a single electroplax are the same as described in preceding papers[1,2].

RESULTS

Table I lists the compounds tested and the M.A.C., *i.e.*, that which causes at least a 50% decrease in the height of the action potential within 30 min or less (usually between 10 and 30 min). The reasons for using this index of activity were discussed previously[1].

Procaine, tetracaine and dibucaine, usually referred to as local anesthetics, are receptor inhibitors[3]. Their potencies upon frog sciatic nerve fibers have been compared and correlated with their lipid solubilities[5-10]. Since they are tertiary ammonium derivatives it will depend upon the pH as to whether the nitrogen atom has a positive charge

TABLE I

MINIMAL ACTIVE CONCENTRATIONS* OF COMPOUNDS WHICH ACT AT THE CONDUCTING MEMBRANE AS WELL AS AT THE SYNAPSE

The first three compounds affected the direct (d) and indirectly (i) elicited responses about equally.

Compounds	*No. of Expts.*	*M.A.C. μg/ml*	*Time (min)*
Procaine	4	125–250	10–20
Tetracaine	3	10–20	10–15
Dibucaine	4	5–10	10–20
PAD	6	5–25	10–30
NorACh 12	3	(d) 50–100	5–20
	3	(i) 5	10–20
NorCh	3	(d) 25–100	20–30
	3	(i) 5	10–20
Atropine	4	(d) 200–2000	20–30
	4	(i) 200	20–30

* Concentration required to decrease spike height by 50% within 30 min or less (see last column).

or not. All drugs listed in Table I were dissolved in Ringer's solution of pH 7.0–7.5.

Fig. 1 shows an example of the results obtained with dibucaine, a much more potent local anesthetic than procaine. Fig. 2 shows the usual response obtained with

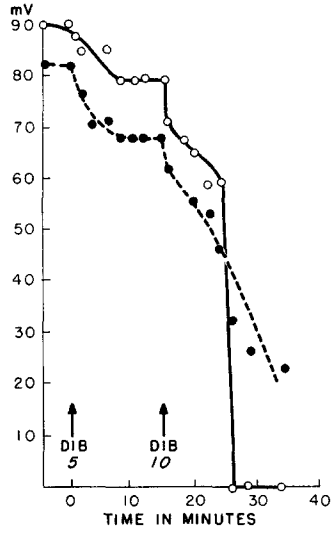

Fig. 1. Effect of DIB on the electrical activity of an isolated single electroplax of *Electrophorus electricus*. The compound was added to the pool bathing the innervated membrane. The response (in mV) to direct ●---● and indirect ○—○ stimulation as recorded by an oscilloscope using extracellular electrodes. The concentrations in μg/ml are indicated by the figures below DIB.

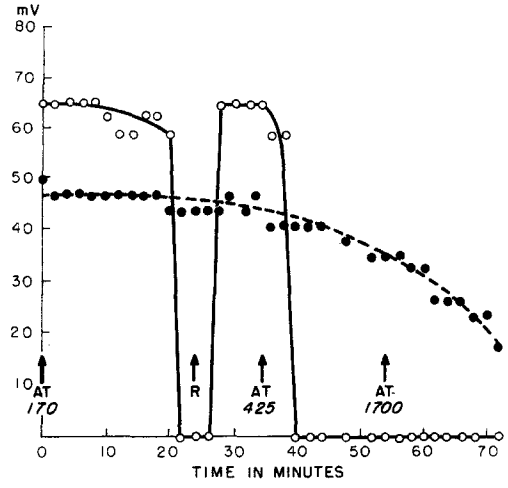

Fig. 2. Effect of atropine (AT) on the electrical activity of an isolated single electroplax. Recordings and symbols as in Fig. 1. The concentrations in μg/ml are indicated by the figures below AT. At R the preparation was washed with Ringer's solution.

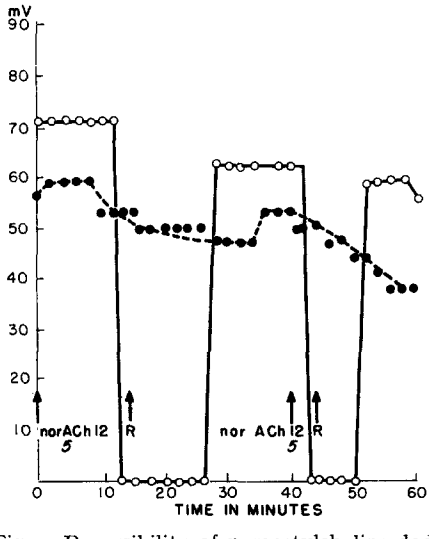

Fig. 3. Reversibility of noracetylcholine dodeciodide action on the electrical activity of an isolated single electroplax. Recordings and symbols as in Fig. 1. The concentrations (μg/ml) are indicated by the figures below norACh 12. At R the preparation was washed with Ringer's solution.

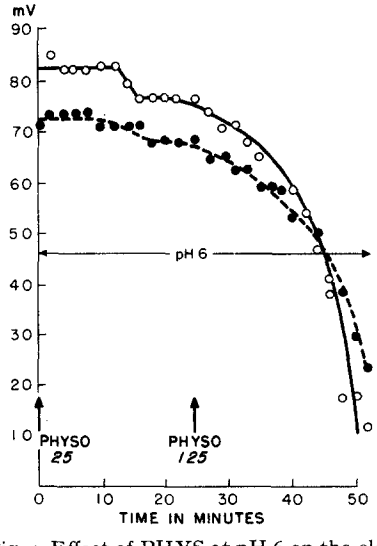

Fig. 4. Effect of PHYS at pH 6 on the electrical activity of the isolated single electroplax.

atropine; *i.e.*, a block of the indirectly elicited spike in a lower concentration than the direct. Complete reversal of block by atropine of indirect stimulation is readily obtained and at least a 50 % reversal of the directly elicited spike. The effects of 5 to 25 μg/ml PAD were usually irreversible although in one case we obtained a 50 % reversal of block of the indirect response. Block of the indirectly stimulated spike by norACh 12 (5 μg/ml) was at least 50 % reversible as shown in Fig. 3. No return of electrical activity occurred after block by 50–100 μg/ml norACh 12 nor any effective concentration of norCh.

Since the acetylcholine receptor is thought to possess an anionic site[3], it was of interest to compare the effect upon electrical activity of the charged *versus* the uncharged form of a tertiary amine. Physostigmine and T.P. were therefore tested at pH 6 and 9. 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 alter the electroplax action potential. Both of the compounds were somewhat weaker at the basic pH as shown in Table II and Figs. 4 and 5. The mean $\pm$ the standard error for the M.A.C. of physostigmine at pH 6 was 280 $\pm$ 76 μg/ml which was significantly less ($P < 0.01$) than the value at pH 9 (890 $\pm$ 253 μg/ml). Under identical conditions neostigmine did not vary in potency with a change of pH from 6 to 9.

TABLE II

EFFECT OF pH UPON THE ACTION OF PHYSOSTIGMINE AND THE TERTIARY ANALOGUE OF PROSTIGMINE

Compound	*pH*	*No. of Expts.*	*M.A.C. µg/ml*	*Time (min)*
Physostigmine	5.5–6.5	5	100–400	10–25
	8.5–9.5	5	200–1300	10–25
Tertiary Analogue of Neostigmine	5.5–6.5	5	1000	20–30
	8.5–9.5	4	2000	15–30

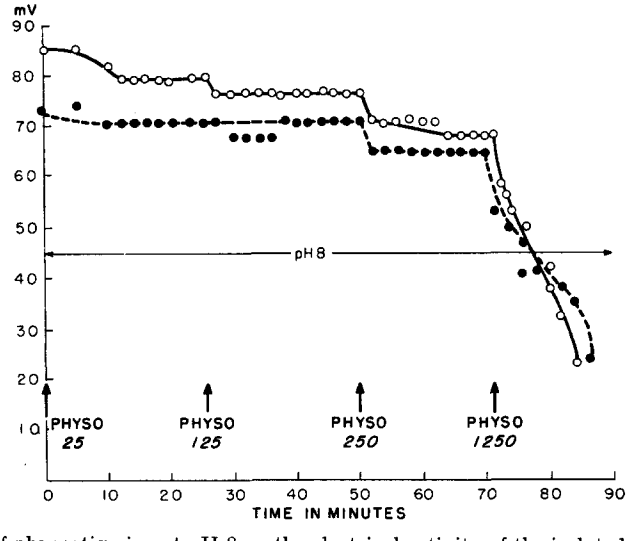

Fig. 5. Effect of physostigmine at pH 8 on the electrical activity of the isolated single electroplax.

All compounds listed in Tables I and II were applied to an electroplax following block of the indirectly elicited spike by 50 μg/ml curare. The compounds to be tested were in all cases combined with 50 μg/ml curare to ensure that the synaptic receptors remained blocked. All were found about as effective in blocking the directly evoked response, following block of indirect response by curare, as they were in the absence of curare, indicating they can act directly upon the conducting membrane. Physostigmine was about 0.5 as potent at pH 8 as at pH 6 in affecting the directly evoked response, which is similar to what was observed in the absence of curare.

DISCUSSION

Tertiary amines

Two types were tested: (a) compounds known to have strong inhibitory action on cholinesterase (physostigmine and the tertiary analogue of Prostigmine), (b) compounds referred to as local anesthetics, presumed to act mainly on the receptor. Physostigmine is also a receptor inhibitor, and blocks electrical activity of the electroplax before the enzyme concentration reaches a critically low level, as was shown by NACHMANSOHN *et al.*[11]. The view that eserine reacts with the receptor protein has recently been confirmed by studies of EHRENPREIS on the acetylcholine receptor protein in solution. He tested by equilibrium dialysis the binding of a variety of compounds to the receptor protein[12,13]. A striking parallelism became apparent between the strength of binding to the protein in solution and the effectiveness on the electrical activity of the isolated single electroplax. Whereas eserine is a potent receptor inhibitor, the affinity of T.P. towards the receptor protein has not yet been evaluated. T.P. is about 100 times weaker as an enzyme inhibitor than its quaternary analogue.

The existence and importance of an anionic site in the active surface of acetylcholinesterase and of the receptor protein has been demonstrated in many ways and discussed elsewhere[3]. Dimethylaminoethyl acetate was shown to be more potent in blocking electrical activity at pH 6 than at pH 9 (see ref. 1). In the acid range it is present in its cationic form, in the alkaline range mostly as an uncharged molecule. Physostigmine was previously shown to be bound to cholinesterase in solution about 16 times better in the charged than the uncharged form[14]. The action on the intact cell as a function of pH has been tested in the present observation. Both physostigmine and T.P. are at pH 6 about twice as potent in blocking electrical activity as at pH 9. This may appear a small difference compared with that observed with the enzyme in solution. However, in experiments on intact cells many additional factors determine the effect. We do not know to what extent the pH in the conducting membrane is altered by the outside solution; it may and probably will be slightly affected, but almost certainly it will not be the same as in the outer fluid. Permeability barriers will favor the action of uncharged molecules. It appears significant that the charged form is nevertheless twice as potent as the uncharged one.

The action of local anesthetics has been attributed to some "tightening" of the conducting membrane. In view of the structural similarity between acetylcholine and local anesthetics NACHMANSOHN has maintained for a long time that they act by competing with acetylcholine for the receptor. The competitive action between procaine and carbamylcholine has been demonstrated on intact electroplax[11]. In recent observations (S. EHRENPREIS AND M. G. KELLOCK, unpublished experiments)

it was found that procaine, tetracaine and dibucaine are bound to the acetylcholine receptor protein in solution. The latter two are much more strongly bound than procaine. It appears pertinent that here again a striking parallelism exists with the effect of the compounds on the intact electroplax. Moreover, tetracaine specifically precipitates receptor protein in contrast to curare which reacts with many other macro-molecules. These developments form a new and strong support for NACHMANSOHN's view on the role of acetylcholine in conduction. The data demonstrate the essentiality of the acetylcholine receptor protein. Considered in combination with the evidence that cholinesterase activity is inseparable from electrical activity, the new facts make it extremely difficult to offer an alternative interpretation.

Atropine blocked the indirect response usually in a lower concentration than required to affect the directly elicited spike. This agrees with the pharmacological observation that atropine in low doses acts primarily at certain synaptic regions. Curare also blocks the indirect response in a much lower concentration than the direct[1], although the concentration difference is much greater than with atropine. This is probably due to the difference in penetration, since when atropine is quaternized and made lipid insoluble by a polymethylene chain, the resulting bis atropinium compound is a potent curarizing agent[15]. Bis atropinium compounds because of their lipid insolubility would not penetrate as well as atropine, and because of their large size might not depolarize[1].

Lipid soluble quaternary ammonium ions

NorACh and PAD, in 10^{-3} *M* concentration, have been found to depolarize the electroplax[4]. In the present observations electrical activity was affected by PAD in about 50 times and norACh in about 10 times lower concentrations than previously reported. However, the criterion used, the minimum active concentration to reduce the activity 50 %, is not quite comparable. Whether the membrane was depolarized has not been ascertained since only extracellular electrodes were used.

Relatively small concentrations of norACh and norCh (5 μg/ml) block the response to indirect but not to direct stimulation. This is the first dissociation observed with these lipid soluble quaternary ammonium ions between the action on synaptic and that on conducting membranes. The concentration acting on the synaptic junction is about 5 times that found with acetylcholine. The concentrations acting on the conducting membrane are about 50 times higher than the concentration of acetylcholine acting on the synaptic junction[1]. This 10 fold difference is relatively small compared to that for lipid insoluble quaternary compounds. The presence of structural barriers surrounding the conducting membrane, whatever their properties, may readily account for some differences in permittivity.

The low concentrations blocking only the synaptic junctions do not seem to depolarize. Depolarization would be expected to block the response to direct stimulation, because in this case the thousands of synaptic junctions per electroplax would produce a short circuiting of the conducting membrane[16]. Thus, low concentrations of these two quaternary ammonium ions seem to act as receptor inhibitors. It is, therefore, interesting that there is virtually no difference between norACh and norCh. Choline is known to be about 2000 times weaker than acetylcholine as a depolarizing agent. It is even weaker than tetramethyl and trimethylethyl ammonium ions. For some reason the oxygen atom may possibly prevent the binding with the receptor. It

is remarkable that when a dodecyl group is substituted for a methyl, the difference between choline and acetylcholine virtually disappears.

PAD, in 5–25 μg/ml, blocks the response to both direct and indirect stimulation simultaneously, suggesting that PAD even in low concentrations may cause depolarization. Such an assumption is supported by the observations of DETTBARN on Ranvier nodes of single sciatic nerve fibers[17].

Although evidence has accumulated that the lipid soluble quaternary ammonium ions in relatively low concentrations, act on conducting membranes by affecting the acetylcholine system, their affinity to the various protein members may differ, dependent on concentrations and on special structural features. They may release acetylcholine from the storage protein, they may act on the receptor either as activators or inhibitors, and finally they may inhibit acetylcholinesterase. Acetylcholinesterase prepared from electric tissue of *Electrophorus* was found to be inhibited *in vitro* about 60 % by 10^{-6} *M* PAD and 70 % by 10^{-4} *M* norCh (HANNA GREENBERG, unpublished experiments). The pertinent fact of the present as well as of previous observations on the action of these lipid soluble quaternary ammonium ions is the evidence that they act on the synaptic and on the conducting membrane in a way similar to other compounds which specifically affect the acetylcholine system.

ACKNOWLEDGEMENTS

We are grateful to Dr. D. NACHMANSOHN for his guidance throughout these studies. Thanks are extended to Dr. SARA GINSBURG who has synthesized several compounds used in the study, and to Ciba Pharmaceutical Products for a generous supply of Nupercaine (dibucaine). We also thank Mr. C. W. COATES for his help in the procurements and maintenance of eels, and Mr. J. ALEXANDER for maintenance of electrical equipment.

This work was supported by the Neurochemistry Training Grant No. 2B-5216 from the Department of Health, Education and Welfare, U.S. Public Health Service, by the Atomic Energy Commission, Contract No. AT(30-1)-1503, and by a gift from the Muscular Dystrophy Associations of America, Inc.

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