BBA 4157

APPARENT DISSOCIATION CONSTANTS BETWEEN CARBAMYLCHOLINE, *d*-TUBOCURARINE AND THE RECEPTOR

HENRY B. HIGMAN*, THOMAS R. PODLESKI AND EVA BARTELS

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

(Received March 26th, 1963)

SUMMARY

The depolarizing action of carbamylcholine on the monocellular electroplax preparation has been analyzed as a function of concentration. The apparent dissociation constant between carbamylcholine and the acetylcholine receptor was found to be $4.4 \cdot 10^{-5}$ M.

The depolarization due to carbamylcholine is inhibited competitively by *d*-tubocurarine. The apparent dissociation constant between *d*-tubocurarine and the acetylcholine receptor was found to be $2.4 \cdot 10^{-7}$ M.

INTRODUCTION

The electric tissue of *Electrophorus electricus* has proven a valuable tissue for the correlation of the chemical and physical events occurring during nerve activity[1]. The method of isolating a single electroplax developed by SCHOFFENIELS[2] affords a suitable means for a quantitative analysis of the effects of neurotropic compounds upon excitable tissues. The complications encountered when multicellular preparations or cells with complex functions are used are greatly reduced by this method in which a single cell, highly specialized in its function, is maintained in an appropriate and well controlled medium.

Quantitative studies of the antagonism between *d*-tubocurarine and substances which depolarize the motor end plate of frog muscle were recently reported by JENKINSON[3]. This paper presents similar studies with the isolated electroplax preparation.

METHODS

The isolated single electroplax of Electrophorus was mounted in the special chamber previously described[2,4,5] and impaled with a microelectrode. The depolarizing effect of carbamylcholine in the presence or absence of *d*-tubocurarine was determined by continuous recordings of the membrane potential. In experiments in which both *d*-tubocurarine and carbamylcholine were employed, the cell was first pretreated with *d*-tubocurarine for 30 min. If resting potentials measured before and after this

* Present address: Departments of Psychiatry and Neurology, L.S.U. School of Medicine, New Orleans, La. (U.S.A.).

treatment were not reproducible to within ± 5 mV, the cell was discarded. The solution was then changed to one containing carbamylcholine plus the same concentration of *d*-tubocurarine. Carbamylcholine was always applied in a continuously flowing solution. This insures that when the transmembrane potential reaches a steady state, the concentration of the compounds in the solution is the same as the concentration of the solution as originally prepared. Careful control studies showed that alterations in the flow rate did not introduce any artifacts into the recordings of the membrane potential.

In all, 130 cells from 24 different electric fish were employed in the study. At the beginning of the study only one depolarization was recorded for each cell. It became apparent, however, that high concentrations of carbamylcholine depolarize to the same steady value whether applied to a cell with a normal resting potential or to a cell already depolarized by a weaker concentration of carbamylcholine. Later in the study several measurements were made on the same cell. The mean steady state potential for a particular carbamylcholine concentration or combination of carbamylcholine and *d*-tubocurarine concentrations was obtained by averaging the results from several cells dissected from at least three different electric fish.

THEORY

When a compound A referred to as an activator induces a biologic response by reacting with a receptor R, we assume that one molecule of the activator reacts with a single site on the receptor. The equilibrium may be written in the form

$$R + A = R - A$$

The dissociation constant K_A, is given by the law of mass action as

$$K_A = \frac{[R]\,[A]}{[R\text{-}A]}$$

Equal responses probably correspond to the occupancy of equal numbers of receptor sites. However, the strength of the response may not be proportional to the fraction of sites occupied by the activator or inhibitor. If the relationship is linear and a maximum response is obtained only when all the sites are occupied,

$$K_A = \left(\frac{r_{max}}{r} - 1\right)[A]$$

where $[A]$ = free concentration of A at equilibrium, r = response produced by $[A]$, r_{max} = maximum response which would be produced if all the receptor sites were occupied by A* (ref. 6).

Thus,

$$r = \frac{r_{max}\,[A]}{K_A + [A]}$$

Theoretically then the response would be hyperbolically related to the activator

* The assumption that the maximum response requires the occupation of all active sites is probably not correct, since it appears more likely that the maximum is reached before

concentration. However, in biologic systems a response is ordinarily obtained only after the activator concentration has reached a certain threshold value[7,8]. Thus

$$(r + a) = \frac{r_{max}\,[A]}{K_A + [A]}$$

where a is constant and may be determined graphically from a simple plot of r *vs.* [A]. Once a is determined, the other constants, r_{max} and K_A, may be obtained by simple mathematical methods.

If a competitive inhibitor is introduced into the system, then the response to a given concentration of the activator will be diminished. If [A'] and [A] represent concentrations of the activator which elicit the same response in the presence and absence respectively of a concentration [I] of the inhibitor, then the dissociation constant for the reaction $R \cdot I \rightleftharpoons R + I$ was shown by GADDUM[9] to be

$$K_I = \frac{[I]\,[A]}{[A'] - [A]}$$

RESULTS

The application of carbamylcholine to the innervated membrane is followed by an exponential decrease of the membrane potential until a constant level is reached dependent upon the carbamylcholine concentration. The time course of the depolarization during a typical experiment is shown in Fig. 1. The initial rate of this

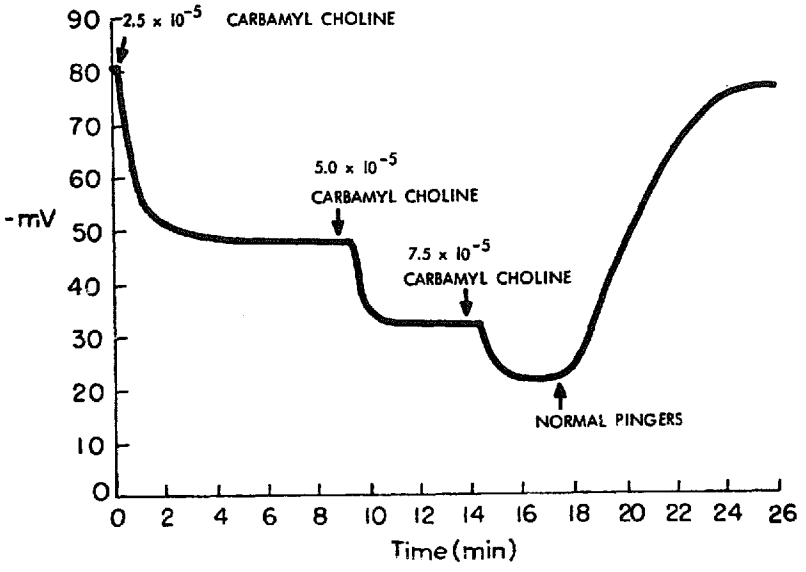

Fig. 1. Effects of carbamylcholine on the monocellular electroplax preparation. The figure presents a typical experiment in which the time course and the degree of depolarization are shown upon addition of increasing concentrations and the repolarization following the return of the cell to RINGER'S solution.

depolarization was not sufficiently reproducible to permit determination of dissociation constants from the kinetic data in the manner of KIRSCHNER AND STONE[7]. However, good reproducibility was found among different cells for the constant level to which the potential fell.

The number of mV of depolarization, *i.e.* mean normal resting potential minus the mean steady state potential following the application of carbamylcholine, is used as the measure of the response to the receptor activator. In Fig. 2, the mean membrane potential is plotted *vs.* the carbamylcholine concentration. The mean resting membrane potential of all the cells employed in the study was —74.9 mV with a standard deviation of ±5.4 mV. When extrapolated to zero activator concentration, the smooth

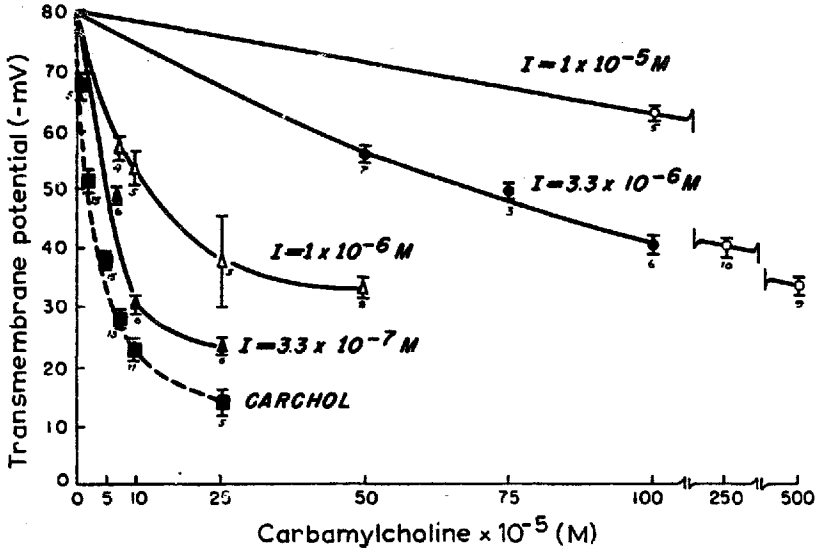

Fig. 2. Competitive action between carbamylcholine and *d*-tubocurarine tested on the electroplax. The points (■) of the lowest curve represent the mean values of the constant potential of the membrane for the concentrations of carbamylcholine given by the abscissa. The number of experiments corresponding to each point is indicated. The degree of depolarization in the presence of carbamylcholine and of receptor inhibitor (I), *d*-tubocurarine, both at varying concentrations indicated in the graph, is a measure of the antagonistic effect. Vertical bars extend for a distance equal to 1 standard error of the mean on either side of the point.

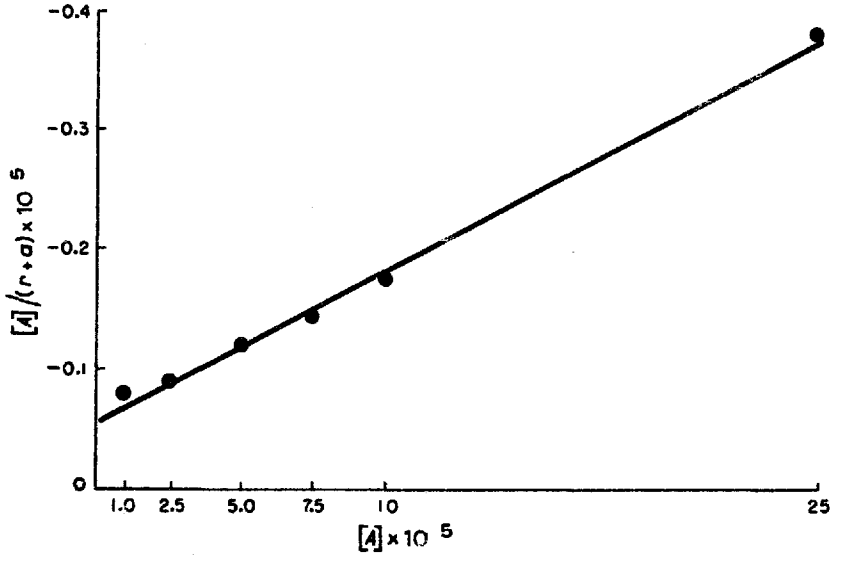

Fig. 3. Apparent dissociation constant between carbamylcholine and receptor calculated as described in the text. The points are taken from the experimental data illustrated by the lowermost curve in Fig. 2. The straight line is fitted by the method of least squares. It has a vertical intercept equal to K_A/r_{max} and slope of $1/r_{max}$.

curve relating the membrane potential to the activator concentration exceeds the normal resting potential by the constant $a = -5.1$ mV.

In Fig. 3, $[A]/(r + a)$ is plotted as a function of [A]. The best straight line passing through the points was determined by the method of least squares. This gives values of −78.5 mV for the maximum depolarization by carbamylcholine and $4.4 \cdot 10^{-5}$ M for the apparent dissociation constant with the receptor.

The inhibition of the depolarization due to carbamylcholine by various concentrations of *d*-tubocurarine is also shown in Fig. 2. Knowing the experimentally determined response to carbamylcholine in the presence of *d*-tubocurarine, the concentration of carbamylcholine required to produce the same response in the absence of *d*-tubocurarine was calculated from the equation

$$[A] = \frac{(r + a)K_A}{r_{max} - (r + a)} = \frac{(r + a)\ (4.4 \times 10^{-5})}{-78.5 - (r + a)}$$

The apparent dissociation constant for the reaction between the receptor and *d*-tubocurarine was then calculated from GADDUM'S equation and found to be $2.4 \pm 1 \cdot 10^{-7}$ M.

DISCUSSION

A number of assumptions were made in the theoretical section which do not appear capable of experimental verification at the present. Some of these, *e.g.* the postulate that the response is linearly related to the number of receptors occupied have been the subject of considerable discussion[10-13]. In the first place, the fraction of receptor sites bound at maximum activation or inhibition is unknown. There may be an excess of available sites over those functionally required. When applied to a cell the compounds used will, moreover, almost certainly react with other unspecific sites in macromolecules by Van der Waals' and electrostatic forces. Secondly, the experimental points in Fig. 3 appear to deviate by a slight concavity from the straight line which fits them best. This may indicate that the response is related to some higher power of the activator concentration. The combination of an O_2 molecule with hemoglobin is "enhanced" by the prior combination of hemoglobin with another O_2 molecule[14]. Similar factors may explain any departure from linearity in Fig. 3. The physiologic significance of the threshold value is not clear. The concentration of the neurotropic compound in the bathing solution may be in a steady state relationship with a somewhat smaller concentration in the still remaining extracellular tissue surrounding the excitable membrane. In view of all these complicating factors it is not possible to refer to the constants determined by these methods as to dissociation constants for the actual chemical reactions between the activator (or inhibitor) and the receptor. However, the method does afford a means of obtaining quantitative data with which to characterize certain chemical effects on cellular function and appears of greater quantitative significance than minimally active concentrations required to produce an arbitrary response, or similar parameters used in the past. The values thus obtained are referred to as "apparent dissociation constant".

GADDUM'S equation for determining the dissociation constant between the inhibitor and the receptor does not involve the assumption of a linear relationship

between number of receptor sites occupied and the response. It only requires that equal responses correspond to the occupancy of equal numbers of receptor sites and that only one activator molecule combines with each site. However, one must determine the concentrations of the activator which produce the same response in the presence and absence of the inhibitor. This is apt to require extrapolation from a smooth curve drawn through experimental points[3]. We feel that greater precision may be obtained through the use of the calculations described above.

The value for the maximum carbamylcholine response is reasonably close to the normal resting potential. This would seem to indicate that a sufficiently high concentration of carbamylcholine might depolarize to zero membrane potential but is not capable alone of causing an overshoot toward the sodium equilibrium potential. This is consistent with the concept that acetylcholine exerts only a "trigger" action[1]. Actually even very high concentrations of carbamylcholine failed to depolarize to zero. The maximum depolarization ever observed with carbamylcholine was to —11 mV. This potential may possibly be determined by impermeable ions. The response obtained with large concentrations of the activator is thus different from the maximum response estimated by extrapolation as has been emphasized by ROCHA E SILVA[11].

The good agreement for this apparent dissociation constant with results obtained by other workers employing other tissues and methods is noteworthy. JENKINSON'S[3] value as determined by depolarization of the motor-end plate of frog muscle is $4.2 \cdot 10^{-7}$ M when calculated as a dissociation constant. VAN MAANEN[15] reported $7.2 \cdot 10^{-7}$ M using a method based on contraction of frog muscle. In a kinetic study of the development of tension in frog muscle, KIRSCHNER AND STONE[7] reported $1.3–3.4 \cdot 10^{-8}$ M for the dissociation constant between the receptor and dihydro-B-erythroidine, a tertiary curare compound.

It is interesting that the inhibitor *d*-tubocurarine should have a smaller dissociation constant than the activator carbamylcholine. The interaction of activators and inhibitors with the receptor is a complex process and involves various factors comparable to those encountered in the reaction between enzyme and either substrate or inhibitor[16].

These observations may be of interest for attempts to isolate the receptor *in vitro*. They suggest that inhibitors with apparently large affinities may be of value in the early stages of isolation although the final identification would involve a specific reaction with an activator.

ACKNOWLEDGEMENTS

We are grateful to Professor D. NACHMANSOHN for his guidance and encouragement throughout these studies. We also thank MR. J. ALEXANDER for maintenance of electrical equipment and MR. C. W. COATES for aid in procurement and maintenance of a supply of electric eels.

This work was supported by the Division of Research Grants and Fellowships, U.S. Public Health Service, Grants 2B-5216 and B-3304, by the National Science Foundation, Grant NSF-G-12901, and by a Gift from the Muscular Dystrophy Associations of America, Inc.

One of the authors (H.B.H.) was a Special Trainee Fellow, U.S. Public Health Service, Grant No. BT-526.

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