THE PHARMACOLOGY OF AN INSECT GANGLION:
ACTIONS OF CARBAMYLCHOLINE AND ACETYLCHELINE

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SUMMARY

1. Methods for presenting dose-response data for the ganglionic actions of cholinergic agonists (e.g. carbamylcholine) are compared, using the mannitol-gap technique for electrophysiological recording of synaptic events at the cercal nerve, giant fibre synapse of the sixth abdominal ganglion of the cockroach Periplaneta americana.

2. At concentrations around $10^{-6}$ M, carbamylcholine has no effect on ganglionic polarization but potentiates the monosynaptic EPSP. At $10^{-5}$ M and higher concentrations, ganglionic depolarization is accompanied by a reduction of EPSP.

3. Pretreatment with eserine ($10^{-8}$ M) considerably shifts the dose-response curve for acetylcholine so that synaptic transmission is consistently sensitive to $10^{-6}$ M acetylcholine.

INTRODUCTION

The overall aim of the present series of experiments is to determine the properties of the acetylcholine receptors in an insect central ganglion. The two main experimental approaches to this problem are biochemical and pharmacological. A biochemical study of the acetylcholine receptor(s) isolated from the ventral nerve cord and the sixth abdominal ganglion of the cockroach Periplaneta americana is currently being undertaken. The present series of pharmacological experiments constitutes a parallel investigation of cholinergic transmission at the cercal nerve, giant fibre synapse located in the sixth abdominal ganglion of Periplaneta americana.

Following the demonstration by Pumphrey & Rawdon-Smith (1937) of a monosynaptic link between fibres in the cercal nerve and giant interneurones in the sixth ganglion of Periplaneta americana, a number of pharmacological studies have been carried out on this synapse (Roeder & Roeder, 1937; Roeder, 1948; Twarog & Roeder, 1957; Yamasaki & Narahashi, 1958, 1960; Callec & Boistel, 1967, 1971; Flattum & Shankland, 1971; Shankland, Rose & Donniger, 1971; Callec, 1972). This work has recently been reviewed, and the overall evidence for cholinergic transmission is compelling when consideration is given to the physical and chemical barriers which

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can reduce the activity of acetylcholine and drugs applied to the ganglion (Callec, 1972, 1974).

Recent advances in techniques for recording synaptic events at the cercal nerve, giant fibre synapse of the cockroach have facilitated the interpretation of results of pharmacological experiments (Pichon & Callec, 1970; Callec & Sattelle, 1973; Sattelle, 1976). In this investigation, the mannitol-gap technique (Callec & Sattelle, 1973) has been employed to compare various ways of expressing the dose response relationship of a cholinergic agonist. In addition, the actions of carbamylcholine (carbachol) and acetylcholine have been investigated.

MATERIAL AND METHODS

The mannitol-gap technique employed to record synaptic potentials from the sixth abdominal ganglion of Periplaneta americana was as described in a previous report (Callec & Sattelle, 1973). Most of the experiments reported here were performed on partly desheathed ganglia in which the sheath was split in several places using fine stainless-steel needles. This procedure improved the accessibility of synaptic elements to drugs introduced into the bathing medium. The normal Ringer solution employed in these experiments had the following composition: NaCl, 208·6 mM; KCl, 3·1 mM; CaCl₂, 5·4 mM; NaHCO₃, 2·0 mM; pH 7·0. Acetylcholine chloride, carbamylcholine chloride and eserine sulphate were dissolved in this Ringer solution.

RESULTS

1. Effects of carbamylcholine (carbachol)

Changes in EPSP and ganglionic polarization

When carbamylcholine chloride was added to the Ringer solution at concentrations of $10^{-4}$ M and higher, a rapid reduction in the monosynaptic EPSP was noted. This was accompanied by a rapid ganglionic depolarization. The time-course of the EPSP changes and the depolarization were followed for several preparations during the application of $5 	imes 10^{-5}$ M carbamylcholine chloride. At this concentration the EPSP was quickly blocked and a depolarization of 25–35 mV was noted. On subsequent re-exposure to normal Ringer, synaptic transmission recovered after some delay, the EPSP and ganglionic polarization being restored (Fig. 1).

In several experiments in which low concentrations (around $10^{-5}$ M) of carbamylcholine were applied, although no change in polarization was noted, a distinct, reversible potentiation of the EPSP was detected (Fig. 2). When longer exposures to higher concentrations of carbamylcholine were studied, repolarization of the ganglion was noted in the presence of the drug. This attenuation of the ganglionic response (desensitization) was clearly seen when $10^{-8}$ M carbamylcholine was applied continuously. Complete repolarization was noted within about an hour but not the return of the EPSP (Fig. 3).

Dose-response curve for carbamylcholine

The response of the ganglion to carbamylcholine was used to evaluate various ways in which the dose-response curve for the ganglionic actions of cholinergic agonists
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Fig. 1. Changes in the amplitude of the monosynaptic EPSP and ganglionic polarization in response to the application of $5 \cdot 10^{-4}$ M carbamylcholine to a desheathed ganglion. Solid line is a continuous recording of ganglionic polarization; (●) EPSP amplitude. White bar denotes period of application of carbamylcholine.

![Graph showing changes in EPSP and polarization](image)

Normal saline

+ carbamylcholine

(5 min)

+ carbamylcholine

(20 min)

(35 min)

Normal saline

(after 20 min)

2 mV

20 ms

Fig. 2. Potentiation of EPSP by the application of carbamylcholine ($10^{-5}$ M) to an intact ganglion.

![Graph showing potentiation of EPSP](image)
could be usefully evaluated. The curves in Fig. 4 were constructed using data obtained from eighteen ganglia. For Fig. 4a all the values for depolarization at a particular concentration were utilized whether the ganglion was challenged once or up to six times with the drug. The mean depolarization and the standard error were calculated for each concentration.

Some problems of utilizing the data in this way to produce a dose-response curve emerged when the effects of repeated doses on the same preparation were examined. In the lower curve of Fig. 4b increasing doses of carbamylcholine were added successively to the same ganglion. After each application, the ganglionic polarization was allowed to recover in Ringer to the initial level before a subsequent (higher) concentration was added. Under these conditions the maximum level of depolarization achieved was usually much less than when a ganglion was challenged only once by a high concentration of carbamylcholine. Similar findings were noted with other drugs (Callec, Dowson & Sattelle, 1975). This attenuation of the response by desensitization could be avoided by challenging each preparation with only one test concentration of carbamylcholine. The upper curve in Fig. 4b was obtained by plotting data from thirteen ganglia which were tested once or twice only. In cases where the preparation was challenged twice, a concentration giving a minimal response was initially employed, followed by a higher concentration giving a maximal or near maximal response. Under these conditions, the response of the ganglion was not noticeably depressed by the application of the first, low, concentration. The resultant curve was steeper and the experimental points were less scattered than in Fig. 4a.

A dose-response curve was also computed from a twin-pulse experiment on a single ganglion following a method previously applied to the response of the isolated rat superior cervical ganglion to acetylcholine in the presence of eserine (Cuthbert & Dunant, 1970; see also Appendix). Such a curve is illustrated in Fig. 4c (solid line). The dose-response curve computed in this way was compared with data points representing thirteen first responses on thirteen separate ganglia (Fig. 4c). The experimental points showed at best only an approximate fit to the computed curve.

For the remainder of this study and in subsequent experiments in this series (see also Callec et al. 1975), dose-response curves have been produced in the manner
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Fig. 4. A comparison of methods for presenting dose-response data for the ganglionic depolarizations induced by various concentrations of carbamylcholine (see text for full details). In (a) results of experiments on 18 ganglia are summarized. The mean depolarization for a particular dose is determined. Vertical bars show twice the standard error. In (b), the upper curve (●) is constructed by plotting results obtained from 13 ganglia which were tested once or twice only; the lower curve (○) is derived from an experiment in which a single ganglion was challenged successively by increasing concentrations of carbamylcholine. Between each test pulse repolarization was observed in normal Ringer. In (c) the dose-response curve computed (see Appendix) from a twin-pulse ($t_1$, $t_2$) experiment on a single ganglion (solid line) is compared with data points representing thirteen first responses on thirteen separate ganglia. All experiments were performed on dasheathed ganglia.
Concentration (µ)

Fig. 5. Dose-response curves (produced from first responses and the results of twin-pulse experiments on desheathed ganglia) for the ganglionic depolarizing actions of acetylcholine (O), carbamylcholine (△) and acetylcholine in the presence of eserine (●).

shown in Fig. 4b (upper curve) using only first responses or the results of twin-pulse experiments in which the test concentrations were widely separated, the first pulse producing a near minimal response. By adopting this method in preference to the other two described, the effects of desensitization were made negligible and a dose-response curve was produced which minimized the scatter of experimental points, allowing a ready comparison of ganglionic sensitivity to a range of applied drugs.

2. Effects of acetylcholine

Changes in EPSP and ganglionic polarisation

The action of acetylcholine was described previously when it was noted that the application of acetylcholine at 4·10^{-8} M to a desheathed ganglion resulted in depolarization accompanied by abolition of the EPSP (Callec & Sattelle, 1973).

Dose-response curve for acetylcholine

A dose-response curve was obtained for acetylcholine using the method outlined for carbamylcholine in which primarily first responses (and first and second responses in the case of a low dose followed by a high dose) were used (Fig. 5). This curve was of similar shape to that described for carbamylcholine but was shifted to the right on the log concentration axis (Fig. 5).

In a number of experiments the desheathed ganglia were pretreated for 10–15 min. with the anticholinesterase eserine at a concentration of 10^{-6} M. The subsequent addition of acetylcholine at 10^{-6} M and higher concentrations, in the presence of eserine,
resulted in a rapid depolarization and reduction in amplitude of the EPSP. The dose-response curve shown in Fig. 5 was obtained. Only a brief pretreatment with the anti-cholinesterase was possible, since prolonged (20–25 min.) exposure to eserine at this concentration produced depolarization.

**DISCUSSION**

The mannitol-gap technique has proved to be a useful way of rapidly obtaining dose-response data for the actions of pharmacological agents on synaptic transmission in the terminal abdominal ganglion of the cockroach (*Periplaneta americana*). In this report, we have followed changes in the monosynaptic EPSP (recorded from the cercal nerve, giant fibre connexions) and ganglionic polarization (cf. Callec & Sattelle, 1973), during the actions of acetylcholine and carbamylcholine. Clearly the response to a drug recorded in this way is several physiological steps away from the primary drug-receptor interaction (cf. Triggle, 1971). Other problems in the interpretation of the results of pharmacological experiments on a central nervous ganglion include multiple sites of action and differential accessibility of structurally related drugs. In *intact* and *desheathed* ganglia and connectives, problems of drug accessibility may be severe. For example, both the lipid solubility and charge of the molecule are important factors in accessibility (O'Brien & Fisher, 1958; O’Brien, 1967; Thomas, 1976a, b).
In the case of acetylcholine and closely related chemicals, the presence of acetylcholinesterase and non-specific choline esterases may profoundly affect the access of the drug (Colhoun, 1958, 1963; Smith & Treherne, 1965). An added complication is that the amplitude of the response to a particular dose of the drug should strictly be assessed under equilibrium conditions. It is nevertheless common practice to record the maximum response following a single dose.

With these limitations in mind, ways of evaluating dose-response data have been considered for the cholinergic agonist carbamylcholine. This drug is highly resistant to hydrolysis by either acetylcholinesterase or non-specific cholinesterase. Data obtained in experiments with carbamylcholine have been plotted in various ways in order to establish criteria for the production of a useful dose-response relationship. One method has been to pool data from several experiments on different ganglia (Fig. 4a). This can distort the dose-response curve for two main reasons. First, averaging the responses at a particular dose-level will flatten the 'mean' curve in comparison with the curve that would be obtained for a single preparation. Secondly, any 'desensitization' of the preparation during the application of repeated doses of agonist will also flatten the dose-response curve. The return to the initial level of polarization in normal Ringer following achievement of a maximum depolarization by a particular dose of agonist is no guarantee of a recovery of full sensitivity (Fig. 4b, lower curve).

In another method of obtaining a dose-response curve it is assumed that the rate of diffusion of carbamylcholine to the receptor governs the time-course of depolarization. Under these conditions a dose-response curve can be computed (based on diffusion equations) using data from the application of a supramaximal and a submaximal dose to a single preparation (see Cuthbert & Dunant, 1970; see also Appendix). A comparison of the curve computed in this way with data points that represent first responses only reveals at best an approximate fit (Fig. 4c). This method has been used to derive a dose-response relationship which is a good fit to data for the response of the isolated rat superior cervical ganglion to acetylcholine in the presence of eserine (Cuthbert & Dunant, 1970). It is not immediately obvious why the assumptions made for the vertebrate ganglion do not appear to hold so well for the insect preparation. However, the successful application of equation 1 (Appendix) demands that the thickness of the stationary layer is small compared to the dimensions of the preparation. This may well not be the case in the insect ganglion with its long and convoluted system of extracellular spaces.

Finally, we have obtained a dose-response curve based on first responses from ganglia and the results of twin-pulse experiments in which the test concentrations are widely separated (Fig. 4b, upper curve). Since this procedure minimizes the effects of desensitization and considerably reduces the scatter of the experimental points by comparison with the other two methods it has been adopted as a routine method for producing dose-response curves in this survey.

The application of a relatively low concentration of carbamylcholine (around $10^{-5}$ M) induces a slight potentiation of the EPSP; higher concentrations give rise to ganglionic depolarization accompanied by either a decrease or block of the EPSP, according to the dose. These last mentioned changes can be accounted for in terms of postsynaptic actions, and the results for carbamylcholine closely parallel those for acetylcholine reported in an earlier paper (Callec & Sattelle, 1973). Presynaptic effects of this drug
have been noted in the sympathetic ganglion (Volle & Koelle, 1961). In this vertebrate preparation it is proposed that carbamylcholine stimulates the release of acetylcholine. It may be that in the insect ganglion presynaptic, as well as postsynaptic, effects of carbamylcholine contribute to the potentiation of the EPSP at low concentrations of carbamylcholine.

In an earlier paper in which the mannitol-gap technique was employed (Callec & Sattelle, 1973) and in all previous studies on the cereal nerve, giant fibre synapse (cf. Callec, 1974), the relative insensitivity of even the desheathed preparation to acetylcholine was noted. We note that eserinization (io⁻⁶ M) shifts the dose-response curve for acetylcholine considerably (Fig. 5) so that effects on polarization and EPSP are noted at concentrations of io⁻⁶ M and above. This finding is consistent with the observations of Shankland et al. (1971) who, using qualitative changes in overall spike activity, note that io⁻⁶ M is the lowest concentration of acetylcholine which reliably gives a 'moderate response' to acetylcholine. It should be noted, however, that these authors also pre-treated ganglia with hemicholinium-3 in order to prevent transmitter synthesis, and that in some cases responses were noted at concentrations as low as io⁻⁷ M and 5·io⁻⁷ M acetylcholine.

The main findings of the present report are the development of a routine procedure for presenting dose-response data for the synaptic actions of cholinergic agonists and a quantitative demonstration of the increased sensitivity to acetylcholine of synaptic transmission at the cereal nerve, giant fibre synapse following eserinization of the desheathed ganglion.

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APPENDIX

In this appendix a brief account is given of the theory and method employed to compute the dose-response curve shown in Fig. 4c. For a more detailed account of this method and a wider discussion of its applications, the reader should consult the original paper of A. W. Cuthbert and Y. Dunant (1970).

Drugs must traverse a stationary fluid layer by diffusion in order to reach the receptors located on the nerve membranes. If the interaction of the drug and the receptor is rapid, then diffusion can be the rate-limiting process determining the time-course of the reaction and possibly also that of the response. If the time-course of the response (e.g. depolarization) produced by an applied drug is diffusion limited, then it is possible to calculate the concentration of drug existing at the membrane at any time following application and thereby relate concentration to response. To achieve this the following assumptions must be made: (i) only a small fraction of nerve cell membrane is occupied by receptors and negligible amounts of the drug are immobilized by the receptors compared to the total amount of drug reaching the cell surface; (ii) all receptors involved in the response are equidistant from the bathing solution (i.e. the stationary layer has a constant thickness l); (iii) the interaction of drug and receptor and the consequent cellular events are very rapid; (iv) the diffusion coefficient of carbamylcholine is assumed to be constant in the stationary layer, which
in this case consists largely of remnants of the neural lamella and the extracellular spaces of the ganglion.

We have no direct measure of $l$ but the dimensionless parameter $Dt/l^2$ can be used (where $D =$ diffusion constant; $t =$ time). Solutions to the equation below for diffusion through a plane sheet can be used when $l$ is small compared to the dimensions of the preparation. Thus:

$$\frac{\delta C}{\delta t} = \frac{D \delta^2 C}{\delta x^2} \tag{1}$$

(where $C =$ concentration; $x =$ distance normal to the plane of diffusion at $x = 0$). Relative concentration ($C(0)/C_0$) is plotted against $Dt/l^2$ (where $C(0) =$ concentration at time $t$ at $x = 0$; $C_0 =$ concentration in the bulk solution). Using data from this curve (see Cuthbert & Dunant, 1970) a series of plots of $l^2/D$ versus $t$ can be produced for various values of $C(0)/C_0$ (Fig. 6). Clearly there is a horizontal line corresponding to the correct $l^2/D$ ratio for a particular experiment. This is obtained by examining a trace of the time-course of a supramaximal depolarization ($t_3$ in Fig. 4c). In addition a submaximal response ($t_1$ in Fig. 4c) is recorded from the same preparation and a horizontal line drawn on the time-course of the supramaximal response at the level of the submaximal response ($X-Y$, Fig. 6). The series of plots of $l^2/D$ versus $t$ are then superimposed on the trace in such a way that, (a) the time of addition of the carbamylcholine corresponds to zero on the time axis ($t = 0$), (b) lines representing the trace of the supramaximal response, the level of the submaximal response and the appropriate concentration ratio ($t_1/t_3$) must intersect at one point (see arrow in Fig. 6). At this point it is assumed that the concentration of drug at the cell surfaces during the supramaximal response reaches that attained finally for the submaximal response. Since we know the time taken to reach a particular concentration, times corresponding to other fractional concentrations can be read. The response to any concentration can be computed by measuring the amplitude of the supramaximal response at the appropriate time, and in this way a dose-response curve can be constructed (e.g. Fig. 4c, solid line) from a twin-pulse ($F_c$, $t < t_1$) experiment.

REFERENCES


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