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ACETYLCHOLINE RECEPTORS: TOPOGRAPHIC DISTRIBUTION AND PHARMACOLOGICAL PROPERTIES OF TWO RECEPTOR TYPES ON A SINGLE MOLLUSCAN NEURONE

BY HERBERT LEVITAN* AND LADISLAV TAUC

From the Laboratory of Neurophysiology, National Institute of Mental Health, Bethesda, Maryland 20014, U.S.A. and the Laboratoire de Neurophysiologie Cellulaire, Centre d'Etudes de Physiologie Nerveuse du Centre National de la Recherche Scientifique, Paris, France

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SUMMARY

1. The iontophoretic application of acetylcholine (ACh) on to identified neurones in the buccal ganglion of the mollusc *Navanax* produced a biphasic or monophasic membrane potential change which was a function of the current intensity and site of ACh application.

2. Low iontophoretic currents, 200 msec in duration, applied to the somatic surface facing the neuropile, caused a monophasic potential change of 6–10 sec duration, which had a reversal potential of about -50 mV, varied with changes in the $[Cl]_o$ of the bathing medium, and was not blocked by the cholinolytics tested.

3. ACh applied more distal to the soma, in the neuropile, produced a 1–3 sec monophasic response whose reversal potential was more positive than -30 mV, varied in amplitude with changes in the $[Na]_o$ of the medium, and was blocked by cholinolytics such as tubocurarine, hexamethonium and atropine.

4. With larger iontophoretic currents a biphasic response could be obtained, depolarization followed by hyperpolarization, which represented a superposition of the above monophasic potentials.

5. The cholinomimetics propionylcholine and butyrylcholine caused a biphasic response like that to ACh. Carbamylcholine and tetramethylammonium also produced a biphasic response but with a more prominent Cl component than that to ACh. Acetyl-β-methylcholine, oxytremorine

* Present address: Behavioral Biology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014.

and pilocarpine only produced a response comparable to the chloride phase of the ACh response.

6. Anticholinesterases prolonged both phases of the ACh response.

7. It was concluded that each of the identified neurones possess two types of cholinoceptive sites, which are pharmacologically distinct, produced different changes in membrane permeability and are distributed differently over the axo-somatic membrane complex.

INTRODUCTION

The post-synaptic receptor molecule with which a particular transmitter substance interacts is not the same in all cases. It has long been recognized, for example, that the transmitter ACh may interact with so-called muscarinic receptors on some structures and nicotinic receptors on others (Dale, 1914). These receptor types have different pharmacological properties and their interaction with ACh may result in different post-synaptic membrane permeability changes. Several types of receptors have also been described for epinephrine (Ahlquist, 1948), serotonin (Gerschenfeld, 1970) and dopamine (Ascher, 1968).

The present report is the result of a study of the characteristics of ACh receptors on identified ganglion cells in a mollusc, *Navanax*. Evidence is presented that each of the identified neurones possess two types of cholinoceptive sites, which are distributed differently over the axo-somatic membrane complex, are pharmacologically distinct, and are responsible for membrane permeability changes to two different ions.

METHODS

Experiments were performed on specimens of the marine gastropod *Navanax inermis*, 7–25 cm in length, obtained from Pacific Bio-Marine Supply Co. (Venice, California). The buccal ganglion, located on the ventral surface of the pharynx, was isolated from the animal, pinned to paraffin in a small lucite chamber and bathed at room temperature (20–25° C). The enveloping connective tissue of one or both halves of the ganglion was cut and reflected, usually with the dorsal surface up. The cell bodies of four neurones (G-L, M-LD, G-R, M-R), identified in a previous study (Levitan, Tauc & Segundo, 1970), then lay exposed on the rostral side of the ganglion, and smaller dorsal cells and neuropile covered the rest of the field. Double-barrelled micropipettes filled with 3 M-KCl were inserted into the soma of one of the four cells. One electrode was used to polarize the membrane, the other to record membrane potential variations. Only electrodes with coupling resistances greater than $10^4\ \Omega$ were used, i.e. in extracellular conditions a current of 100 nA through one barrel produced a voltage change of less than 1 mV in the other. Since the input resistance of the cells under investigation ranged from about 0·5–1·5 MΩ, a current of 100 nA produced a minimum membrane potential change of 50 mV. Thus utilizing double-barrelled electrodes with coupling resistances of more than $10^4\ \Omega$ introduced less than a 2 % error in the determination of membrane potential. Input resistance of the cells was monitored by passing 2–5 nA pulses of current through the

polarizing electrode and monitoring the membrane potential changes thus produced. The intracellularly recorded signals were lead off by unity gain cathode followers with negative capacitance compensation and displayed on an ink recorder (Brush Model 280) and oscilloscope (Tektronix 502).

Cholinergic drugs were applied as cations by iontophoretic pulses (del Castillo & Katz, 1955), 200–300 msec in duration, from single or double-barrelled micropipettes containing salt solutions at concentrations of 0·1–0·2 g/ml. When cholinomimetics were applied, double-barrelled micropipettes were used, one barrel always being filled with ACh. A braking current of 5–10 nA was applied to both barrels to minimize drug leakage.

The ganglion was normally bathed in Atlantic Ocean sea-water from the bay near Arcachon, France, or in commercially available sea 'water (Marine Magic, by Lambert–Kay, Los Angeles, California), to which was added 100 mM-Mg in the form of $MgCl_2$ or $MgSO_4$. The presence of approximately three times the normal Mg concentration effectively blocked ongoing and drug induced synaptic activity, thereby ensuring that the pharmacological effects were exerted directly on the penetrated cell membrane and not mediated through chemical synapses.

The physiological solutions listed in Table 1 were used to study the nature of the membrane permeability changes produced by ACh application.

TABLE 1. Composition of solutions

	NaCl (m-mole/l.)	Na propionate (m-mole/l.)	KCl (m-mole/l.)	$MgCl_2$ (m-mole/l.)	$MgSO_4$ (m-mole/l.)	$CaCl_2$ (m-mole/l.)	Tris pH 7·8 (m-mole/l.)
Solution A	492	—	10	82	65	11	10
Solution B	—	—	10	121	26	11	600
Solution C	—	492	10	82	65	11	10

Tris (hydroxymethyl) aminomethane was adjusted to pH 7·8 by adding HCl.

The external Na concentration was varied by mixing solutions A and B in varying proportions. Variations in external Cl concentration were accomplished by mixing proportional amounts of solutions A and C. For each case the chloride concentration was determined following the technique of Schales & Schales (1941) for titrating biologic fluids with acid mercuric nitrate solution in the presence of s-diphenylcarbazone as an end-point indicator. Potassium was varied isotonically with Na in solution A when it was desired to maintain a constant chloride concentration, and hypo- or hyperosmotically when a constant Na concentration was desired. Calcium was varied by including more or less than the normal amount of its salt in solution A without changing the concentration of the other constituents. Unless otherwise specified, all solutions contained a 100 mM excess of Mg.

RESULTS

Topographic distribution of ACh receptors

Soma: insensitive to ACh on outer surface. The cell bodies of the identified neurones have diameters of 150–600 μ so that the ACh sensitivity of different regions could be determined with relative ease. The outward facing surface

of all these cells was insensitive to ACh. Iontophoretic currents in the 100 nA–1 μA range, with the ACh electrode in close proximity to the soma surface, produced no detectable change in somatic membrane potential. If the ACh electrode touched or entered the cell, the voltage change produced by iontophoretic current had a duration approximately equal to that of the applied current, reversed in polarity as the applied current was reversed, and was clearly a result of the applied current rather than a response to the drug.

Neuropile. When the dorsal surface of the buccal ganglion was widely opened the inner surface of the identified soma, i.e. that facing the neuropile, became accessible for exploration with the ACh probe. The region close to the inner somatic surface and the neuropile was very sensitive to ACh. Membrane potential changes could readily be elicited with applied currents in the 1 nA or tens of nA range.

ACh applied to the neuropile of the contralateral half of the ganglion was also effective. Since the large cells in both halves of the ganglion are coupled electrically (Levitan *et al.* 1970), a polarization change in any one may be seen in another. The potential change seen in any cell with applied ACh thus represented the response of the monitored cell, with contributions from electrically coupled cells. A map of the ACh sensitive regions of the neuropile would therefore be more indicative of the network of electrically coupled cells than of the axon processes of a particular cell, so that the axon trajectory of a particular cell could not be derived from its responsiveness to ACh.

Application of ACh to the neuropile frequently excited other neurones some of which made chemically mediated synaptic contact with the cells being monitored. In many cases the synaptic bombardment provoked by ACh was so intense that it was not possible to determine the time course of the ACh response of the monitored cell, due to superposition of synaptic activity, or be certain there was in fact any direct ACh response (Fig. 1). To eliminate secondary effects of ACh application due to synaptic bombardment of the monitored cells, 100 mM-Mg was added to the perfusion medium in the form of $MgCl_2$ and/or $MgSO_4$. This effectively blocked both ongoing and ACh evoked synaptic activity to the monitored cells within 2–5 min, and left only the direct response to ACh (Fig. 1). In all subsequent work concerning the response of these cells to iontophoretic application of ACh, a bathing medium containing approximately 150 mM-Mg was used. This is approximately three times the Mg concentration found in natural sea water.

Response to ACh. The most frequent response to a brief iontophoretic application of ACh in the neuropile was a polyphasic potential change (Fig. 2). At the normal resting level of about -60 to -65 mV the potential

was positive-going and the phases were sometimes distinguished by an inflexion in the falling phase of the response. When the steady membrane potential was made more positive than about -45 mV application of ACh produced a clearly biphasic response, usually a positive-going potential, Phase I, followed by a negative-going potential, Phase II. For an ACh current of 200 msec duration, Phase I had a duration of from 1 to 3 sec and Phase II up to 10 sec beyond that.

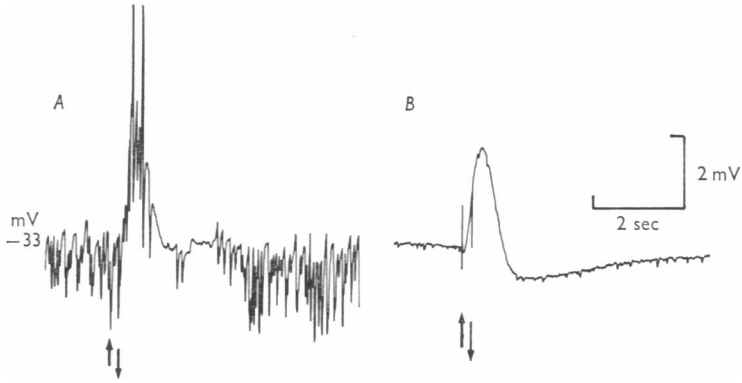

Fig. 1. Iontophoretic application of ACh (arrows) may provoke membrane potential changes in the monitored neurone by direct interaction with receptors on the neurone and by exciting interneurones which have synaptic connexions with the monitored cell. In *A* the ganglion was bathed in normal sea water, and ACh applied while the membrane potential was artificially set at -33 mV, a level more positive than the reversal potential for most of the synaptic activity. The ongoing spontaneous synaptic activity and that provoked by ACh were superimposed to an unknown degree upon the direct response to ACh. In *B*, 100 mM-Mg have been added to the sea water as $MgSO_4$, and ACh applied again, 4 min later. The synaptic activity has been largely suppressed by the addition of Mg and the direct response to ACh revealed. Example taken from cell G-R.

Variation in ACh response with displacement in neuropile and iontophoretic current. The ACh response could be altered either by moving the ACh probe in the neuropile or by changing the intensity of iontophoretic current. For a constant ACh current, displacement of the ACh electrode within the neuropile could change the character of the response as shown in Fig. 3. With the membrane potential held constant at -49 mV, the ACh electrode was advanced in 100 μ steps in a dorsal-ventral course through the neuropile. The response near the dorsal surface of the ganglion was negative-going, but as the ACh electrode advanced, this negative potential was preceded by a positive-going potential, which increased and

then decreased in amplitude as the ACh electrode was moved ventrally. Before disappearing entirely the response became negative-going again. Changes in response were observed as one moved in any direction through a field in the neuropile.

At any particular site in the neuropile alterations in the intensity of iontophoretic current could alter the character of the response at constant membrane potential. In the example shown in Fig. 4, small ACh currents produced a purely negative potential, but as the current intensity was

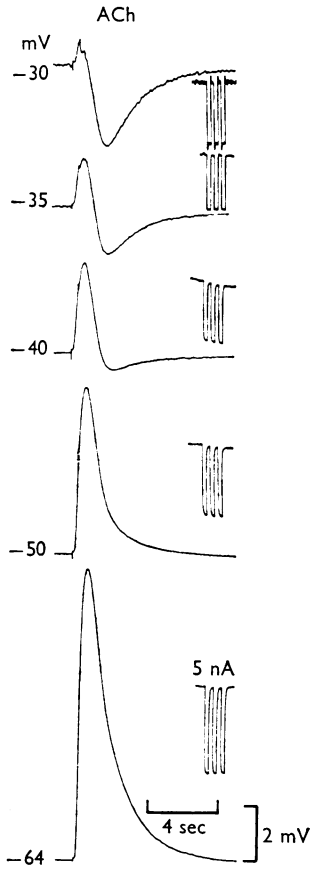

Fig. 2. Biphasic response to 200 msec application of ACh (40 nA). At the resting potential of −64 mV a monophasic depolarization was seen. At −40 mV the initial, depolarizing Phase I was followed by a hyperpolarizing Phase II. The reversal potential for Phase II was about −50 mV. In this and subsequent Figures the series of three pulses to the right of each recording indicates the voltage response to constant current pulses (5 nA in case shown) and their amplitude is thus a measure of membrane resistance. Example from cell G-L in high Mg medium.

increased this was preceded by a positive-going potential. Both phases increased in amplitude with iontophoretic current, up to a saturation level. The current required for maximum response of each phase was not necessarily the same. The example illustrated in Fig. 4 shows a predominantly

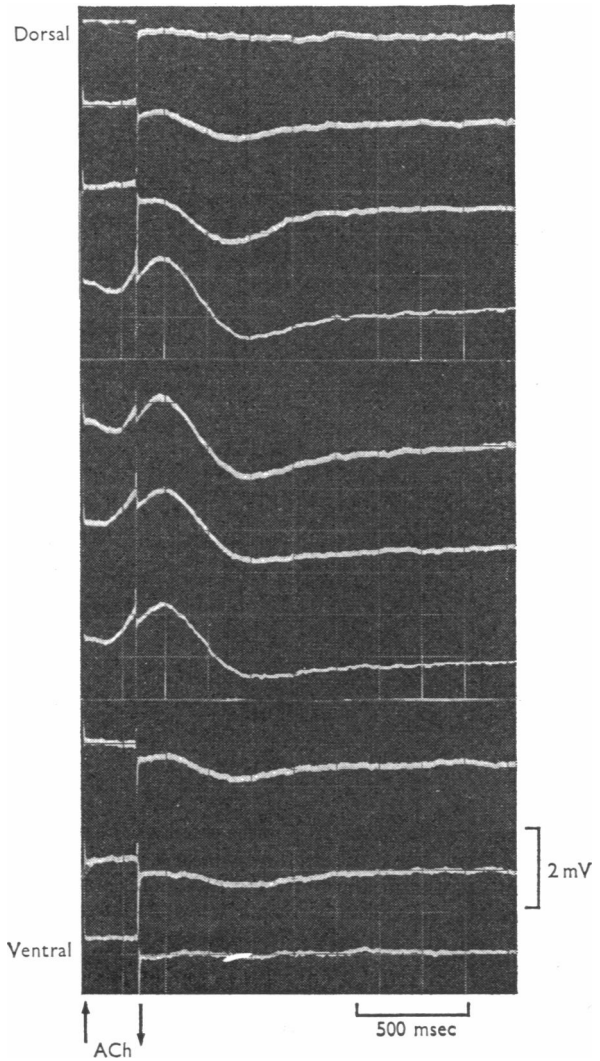

Fig. 3. The character of the ACh response varied as the electrode containing the drug was moved through the neuropile. With the amplitude of iontophoretic current (arrows at bottom) held constant the ACh electrode was advanced in 100 μ steps in a dorso-ventral course through the neuropile. The membrane potential was held constant at -49 mV. Example from cell G-L in high Mg medium.

Phase II response for low iontophoretic currents. In other areas of the neuropile Phase I was predominant at low iontophoretic currents.

Separation of phases topographically. Although a diphasic response could be observed by applying ACh throughout the neuropile of both halves of the ganglion, under some conditions only one phase could be observed. If

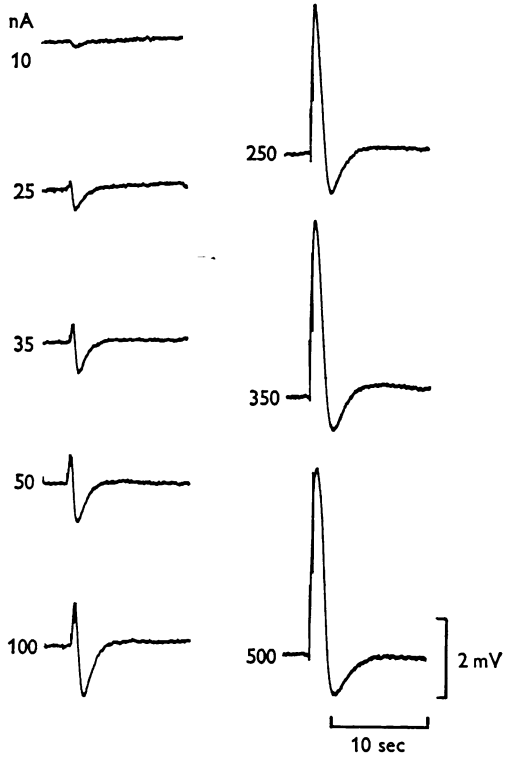

Fig. 4. The character of the ACh response varied as the intensity of iontophoretic current varied. With the ACh electrode held in a fixed position in the neuropile, and the membrane potential held constant at −45 mV, low iontophoretic currents produced a response in which a negative-going, Phase II response was predominant. Higher currents caused a large positive-going, Phase I component to be superimposed. Example from cell M-R in high Mg medium.

the iontophoretic current was reduced to the 1 nA range and ACh applied close to the inward facing somatic surface or near the highly pigmented parts of the soma (regions from which axonal processes were sometimes seen to emerge), a small response could be discerned which had a reversal potential of about −50 mV. The flatness of the response at the reversal potential suggested that this was a pure Phase II response (Fig. 5). As

shown in the Figure the response increased in amplitude as the membrane potential was made more positive or negative than the reversal potential.

When low intensity ACh currents were applied in the neuropile more distal to the soma, a purely depolarizing response of 1–3 sec duration could be obtained (Fig. 6). In many instances the absence of a negative-going component even at membrane potentials much more positive than the

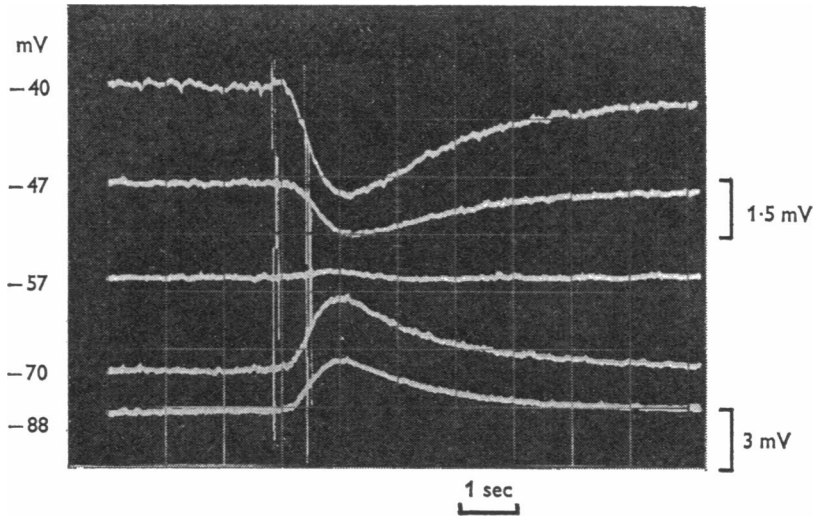

Fig. 5. Small iontophoretic currents close to the inward facing somatic membrane often produced a monophasic response with a reversal potential between −50 and −60 mV; i.e. a purely Phase II response. Voltage calibration of 3 mV applies only to lowest trace. Example from cell M-LD in high Mg medium.

reversal potential for Phase II suggested that in certain areas of the neuropile a purely positive-going Phase I-type response could be elicited. Estimates of the reversal potential for this phase, obtained by extrapolation of the relation between response amplitude and membrane potential to zero response amplitude (cf. Fig. 13), yielded values between 0 and −25 mV.

Pharmacological properties of ACh receptors

Cholinolytics. The two types of response to ACh could also be distinguished pharmacologically. The substances listed in Table 2 were either partially or totally effective in blocking Phase I within 2–10 min of their addition to the perfusion medium, with minimal effect on Phase II. Decamethonium (0·2 m-mole/l.), hemicholinium (0·2 m-mole/l.), probantine (0·2 m-mole/l.) and tetraethylammonium (0·6 m-mole/l.) in the

concentrations indicated had their primary affect on Phase I, but at higher concentrations substantially reduced the total membrane resistance. Atropine (1·5 m-mole/l.), benzoquinonium (1·5 m-mole/l.), dihydro-β-erythroidine (3·5 m-mole/l.), hexamethonium (3·5 m-mole/l.), D-tubocurarine (1·5 m-mole/l.) and gallamine (1·0 m-mole/l.) had little or no effect

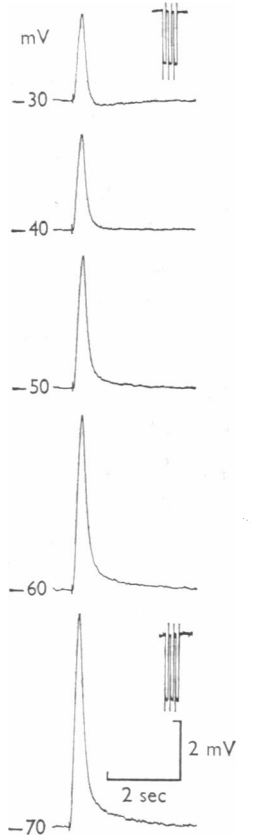

Fig. 6. Small iontophoretic currents applied in the neuropile some distance from the soma produced a monophasic response having a reversal potential much more positive than −30 mV. Example from cell G-R in high Mg medium.

TABLE 2. List of cholinolytics which when added to the perfusion medium of the ganglion selectively blocked Phase I of the ACh response

Atropine	Hemicholinium
Benzoquinonium	Hexamethonium
Decamethonium	Methylxylocholine (βTM 10)
Dihydro-β-erythroidine	Pro-banthine
Gallamine (Flaxedil)	Tetraethylammonium
D-tubocurarine	

on membrane resistance at the concentrations indicated and produced complete blockade of Phase I at these concentrations.

Fig. 7 shows the effect of atropine and dihydro-β-erythroidine on a biphasic response elicited by ACh. In each case the drugs blocked Phase I of the response within 6–10 min, leaving a response which had a reversal potential of about -50 mV, Phase II. Such an effect was typical of the group of drugs listed in Table 2. The initial response was recovered about 30 min after washing out dihydro-β-erythroidine, but even 90 min after the removal of atropine it was only about 50 % recovered.

In Fig. 8 a response is shown (Con) to ACh applied in the neuropile some distance from the soma. It diminished in amplitude with depolarization, remained monophasic and positive-going up to -30 mV, and thus exhibited all the characteristics of a predominantly Phase I response. The reversal potential, obtained by extrapolation, was about -20 mV. The addition of

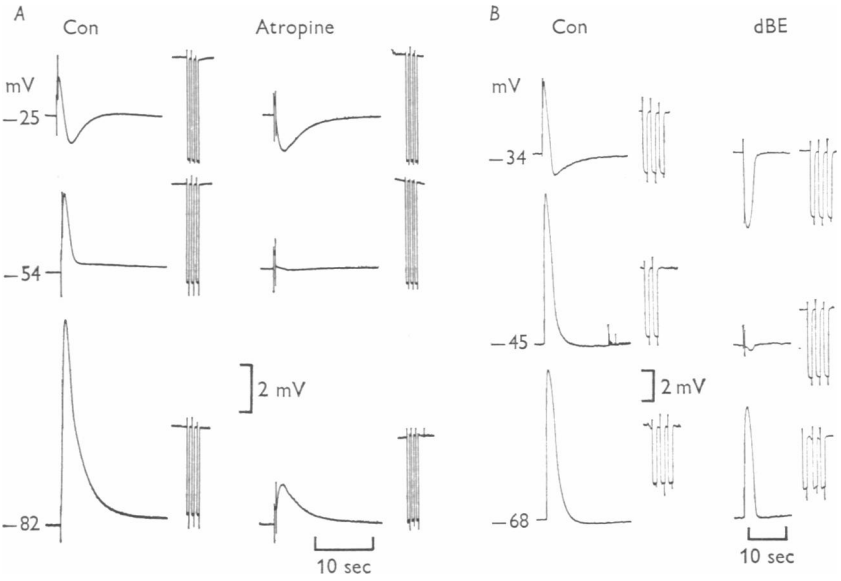

Fig. 7. Effect of cholinolytics on the biphasic response to a 200 msec iontophoretic application of ACh. *A*, control response is shown in left column (Con) at membrane potentials of -25, -54, -82 mV. The response 10 min after perfusing with atropine sulphate (0·75 m-mole/l.) is shown to the right at the same membrane potentials, and demonstrates that atropine blocks predominantly the positive-going, Phase I component of the response. At -54 mV, close to the reversal potential for the Phase II component, no response is seen under atropine. Example from cell G-R. *B*, control responses are shown at left (Con) at -34, -45, and -68 mV. In dihydro-β-erythroidine (dBE, 1·8 m-mole/l.) Phase I is completely blocked, revealing a response with a reversal potential near -45 mV. Example from cell G-L. Voltage calibration is 4 mV for control at -68 mV.

hexamethonium, a potent antagonist for Phase I, reveals the presence of another, hexamethonium-resistant, component with a reversal potential between −30 and −50 mV, presumably a Phase II type response elicited far from the soma.

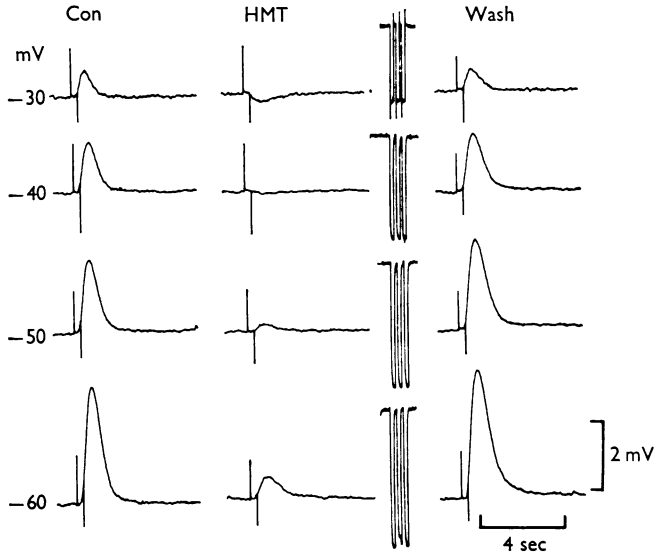

Fig. 8. A monophasic ACh response which was depolarizing at −30 mV (left column) was shown to consist primarily of a Phase I component with the addition of hexamethonium (HMT, 0·7 m-mole/l.), a specific Phase I antagonist. After 2 min in HMT a small, HMT-resistant, response remained with a reversal potential between −30 and −50 mV. Recovery was complete 30 min after washing out this drug. Membrane resistance was unaffected by HMT. Sample from cell G-R.

Effect on Phase II. None of the cholinolytics selectively blocked Phase II of the ACh response. Curare and atropine partially attenuated the amplitude of Phase II when employed in concentrations (1·5 m-mole/l.) which were two to five times that required to obtain complete blockade of Phase I. Hexamethonium had no effect on Phase II at more than twice that concentration (3·7 m-mole/l.) (Fig. 9).

Cholinomimetics. Since none of the wide spectrum of cholinolytics were capable of completely suppressing Phase II of the ACh response, we thought it necessary to characterize the receptor involved and thereby reaffirm its cholinergic nature. This was done by testing the effect of iontophoretic application of cholinomimetics.

The cholinomimetics were iontophoretically applied as cations from a double-barrelled micro-electrode, whose other barrel contained ACh. In this way we could compare the responsiveness of the same region of the neurone to ACh and a cholinomimetic.

Butyrylcholine (BCh) and propionylcholine (PCh) mimicked both phases of the ACh response and differed only in that they produced a slightly longer duration response. This was presumably due to the relative specificity of the cholinesterase in the ganglion for ACh.

Carbamylcholine (CCh) and tetramethylammonium (TMA) elicited responses which, in general, differed considerably from the ACh response. In regions of the neuropile where ACh elicited a biphasic response or one in which Phase I was dominant both CCh and TMA elicited a response which was practically monophasic, the predominant component having a reversal potential around -50 mV (Fig. 10). A positive-going component, comparable to Phase I in the ACh response, could sometimes be detected at membrane potentials close to the reversal potential for Phase II. The duration of the response to these drugs was longer than the ACh response, again due probably to their immunity to hydrolysis by the cholinesterase.

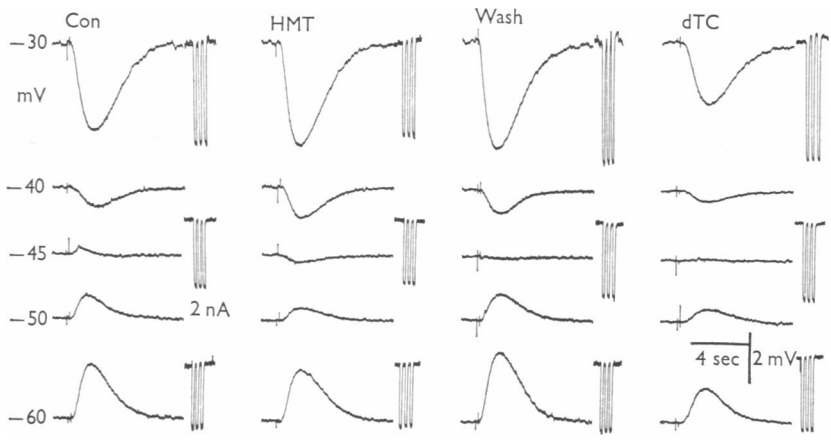

Fig. 9. A monophasic ACh response with a reversal potential near -45 mV was essentially unchanged by the addition of either hexamethonium (HMT, 3·7 m-mole/l.) or curare (dTC, 1·5 m-mole/l.). Control responses at membrane potentials of -30, -40, -45, -50 and -60 mV are shown in left column, followed by the response to 2 nA current pulses to indicate membrane resistance. Second column from left are responses after bathing for 8 min in HMT chloride. The third column are responses 30 min after washing out the HMT, a time normally sufficient to recover from the cholinolytic effects of this drug. The fourth column are the responses after 10 min in dTC. Though attenuated by curare the basic character of the response was unaltered. Sample from cell M-LD.

Acetyl-β-methylcholine (MeCh), oxytremerine and pilocarpine all produced a monophasic response with a reversal potential near -50 mV, no matter what the nature of the ACh response. Responses were most readily elicited with this group of drugs in regions in which Phase II contributed significantly to the ACh response. MeCh produced a shorter duration response than ACh and sensitive regions were more restricted, generally limited to regions of the neuropile close to the cell body.

Cholinolytics which were effective in blocking Phase I of the ACh response were also effective in blocking the corresponding positive-going response in BCh, PCh, CCh and TMA. They were ineffective in attenuating to any extent the response to

MeCh. In the presence of cholinolytics the reversal potential for the remaining monophasic potentials were identical for ACh and all the cholinomimetics, usually about −50 mV.

Cholinesterase inhibitors. Eserine is known primarily for its action as a cholinesterase inhibitor but it may also exhibit 'curarizing' properties (Tauc & Gerschenfeld, 1962). When eserine sulphate (physostigmine) (1 m-mole/l.) was added to the medium perfusing the buccal ganglion its

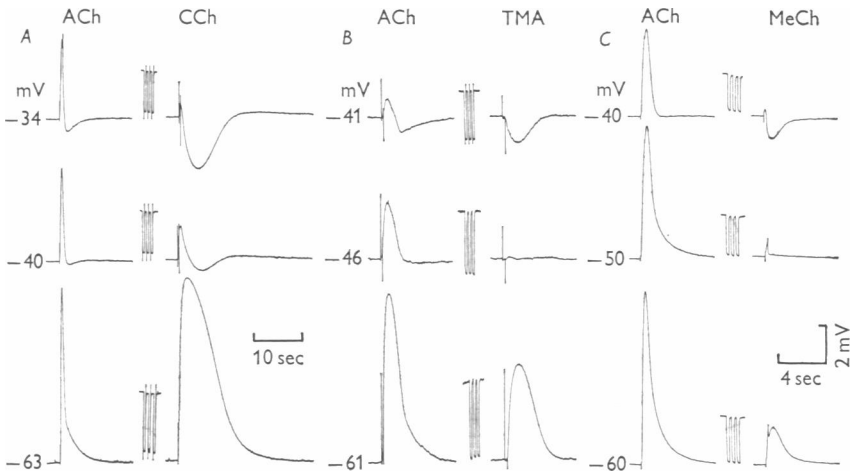

Fig. 10. The response of particular membrane regions to acetylcholine (ACh) was compared with the responses to cholinomimetics by iontophoresing the ACh and the cholinomimetic through separate barrels of a double-barrelled micro-electrode: *A*, carbamylcholine (CCh), *B*, tetramethylammonium (TMA), and *C*, acetyl-β-methylcholine (MeCh). CCh evoked a biphasic response where the ACh response was biphasic, but the negative-going, Phase II response is more pronounced in the CCh response. MeCh and TMA evoked only monophasic, Phase II responses. *A* and *B* are from cell G-R in different animals, *C* from cell G-L in a third animal. Time calibration of 4 sec applies to *B* and *C*; 10 sec calibration to *A*. 2 mV voltage calibration applies to *A*, *B* and *C*.

action on the biphasic ACh response was twofold (Fig. 11). In 6–10 min it almost completely blocked Phase I, leaving Phase II, which, though greatly prolonged, had a clear reversal potential near −45 mV. Eserine thus mimicked the effect of curare in blocking Phase I. The prolongation of Phase II reaffirmed the cholinergic character of this phase and the presence of a cholinesterase in the ganglion.

The 'curarizing' action of eserine was readily reversible by washing, but the anticholinesterase action was not. Thirty minutes after washing out eserine a large, prolonged Phase I was seen superimposed upon an already augmented Phase II. It is then clear that by inhibiting the ganglion

cholinesterase both phases of the ACh response are increased in size and duration.

Eserine had no effect on the response produced by the cholinomimetics (CCh and TMA) but did increase the size and duration of the monophasic MeCh response.

Prostigmine and tensilon are cholinesterase inhibitors which do not have curarizing properties, and their presence readily prolonged both phases of the ACh response.

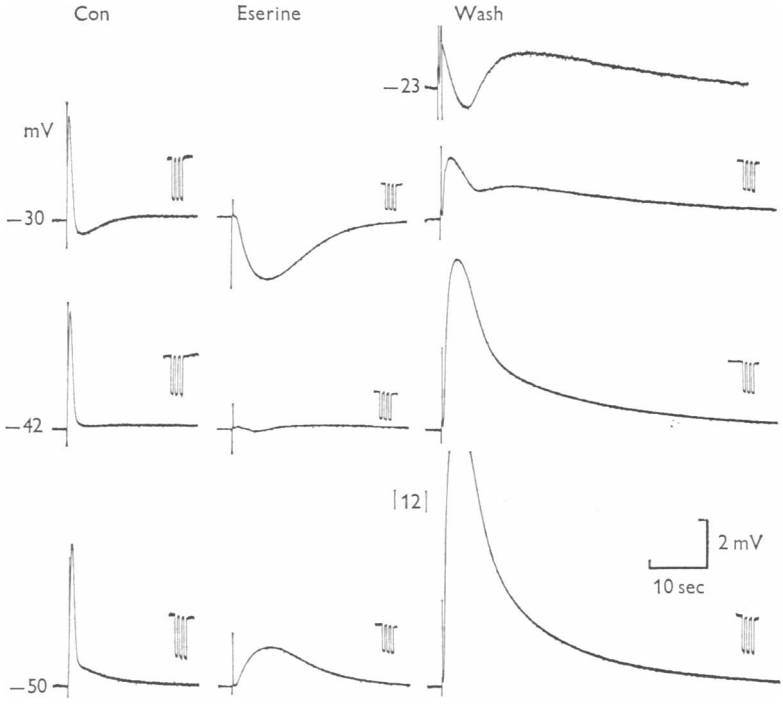

Fig. 11. Eserine had a 'curarizing' and anticholinesterase effect on the biphasic ACh response. In the left column are the control responses to a 200 msec, iontophoretic application of ACh at membrane potentials of −30, −42, and −50 mV. After 20 min in eserine sulphate (1 m-mole/l.) the positive-going, Phase I response was abolished leaving a prolonged Phase II response which had a reversal potential near −42 mV (second column). One hour after washing out the eserine (right column) Phase I recovered and was greatly augmented in both amplitude and duration, demonstrating the reversible character of the 'curarizing' action but irreversibility of the anticholinesterase action. Inflexions in the recovered response at −30 mV were due to superposition of the negative-going Phase II component, shown more clearly at −23 mV. Response at −50 mV is shown truncated at its peak (right bottom) but reached an amplitude of 12 mV, twice that of the control.

Ions involved in permeability changes

Phase II. By using cholinolytics such as D-tubocurare (DTC) or atropine selectively to block Phase I of the ACh response, the changes in membrane permeability associated only with Phase II could be studied. As the Cl concentration was decreased, using propionate as the Cl substitute, the reversal potential for Phase II became more positive (Fig. 12). The reversal potential was unchanged by varying the K concentration from one tenth to five times normal, or reducing the Na concentration to zero (solution B), while maintaining a relatively constant Cl concentration. The reversal potential of Phase II of the cholinomimetic's response and the response to MeCh varied with Cl in the same manner as ACh.

We concluded therefore that Phase II was due to a transient increase in the membrane permeability to Cl.

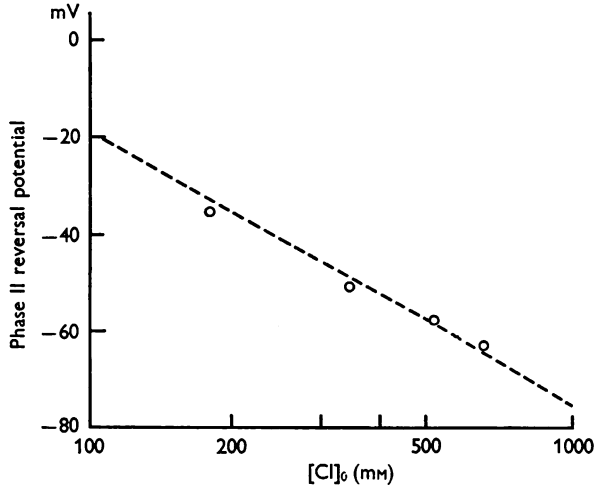

Fig. 12. In the presence of 0·75 m-mole/l. atropine the reversal potential for the remaining, Phase II of the ACh response became more positive with decreasing concentrations of external Cl, $[Cl]_o$. The dashed line has a slope of 58 mV/tenfold change in Cl concentration. Propionate was the chloride substitute in this example from cell M-R.

Phase I. The changes in membrane permeability associated with Phase I of the ACh response were examined by determining the variation in amplitude of Phase I with changes in the ion concentration of the perfusion medium. Since a specific blocker of the chloride dependent Phase II was unavailable, the relative contribution of this component to an ACh response was unknown. In the monophasic response shown in Fig. 8, for example, a small contribution from Phase II was revealed only after application of a specific Phase I blocker. An attempt was made therefore to

maintain a constant $[Cl]_o$, and thus constant Phase II contribution, as other ions were varied. Fig. 13*A* is a plot of the variation in amplitude of Phase I with membrane potential for $[Na]_o$ of 492, 396 and 246 m-mole/l. (solutions A and B). The apparent reversal potential of Phase I for a particular $[Na]_o$ was taken as the value of the regression line at zero

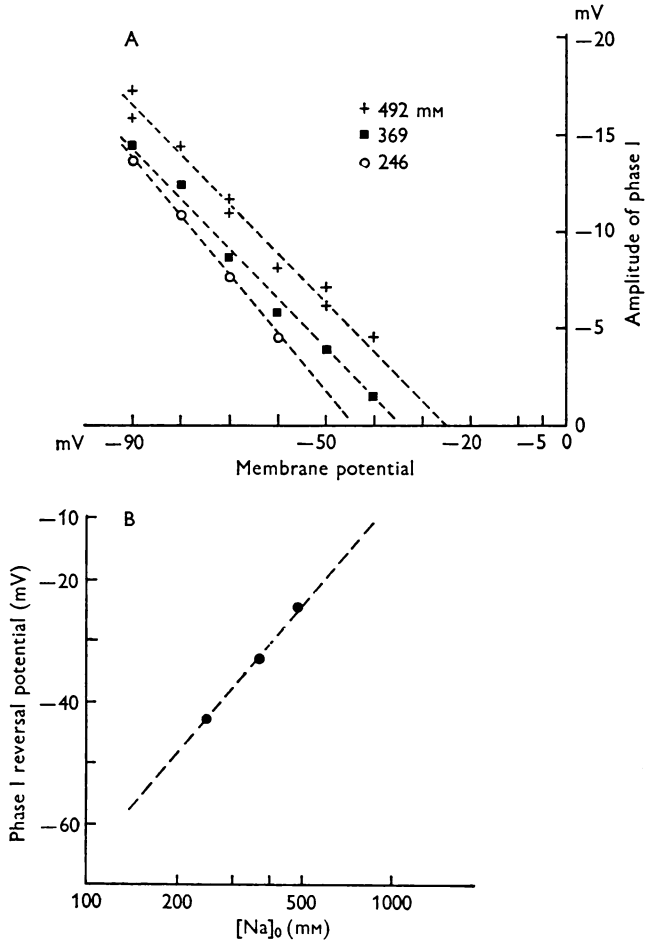

Fig. 13. The dependence of Phase I on Na was demonstrated by determining how the apparent reversal potential, determined by extrapolation, varied with the Na concentration in the perfusion medium. In *A* the reversal potential of Phase I in 492, 369 and 246 m-mole Na/l. was determined by extrapolating the regression line relating response amplitude to membrane potential to zero response amplitude. In *B* the reversal potential for various concentrations of Na are plotted as a function of external Na concentration. The dashed line has a slope of 58 mV/tenfold change in Na concentration. Sample from cell G-L.

amplitude. These apparent reversal potentials are plotted as a function of $[Na]_o$ in Fig. 13*B*, and are seen to fall close to the dashed line whose slope is 58 mV/tenfold change in $[Na]_o$. Variations in the $[K]_o$ and $[Ca]_o$ had no effect on the apparent reversal potential of Phase I in normal or low $[Na]_o$.

It was concluded therefore that Phase I was the result of a transient increase in the membrane permeability to Na. The apparent reversal potentials of 0 to -25 mV obtained in normal saline were presumably a consequence of our inability to readily obtain a pure Phase I response, and reflected the superposition of a chloride permeability change associated with Phase II.

DISCUSSION

In this study of the properties of cholinergic receptors in *Navanax* we have shown a variation in the character of the ACh response as a function of the site of iontophoretic application of the drug. At many sites the response had two phases, with different reversal potentials. The relative weight of each phase varied with the intensity of iontophoretic current or with slight changes in site, and one phase or the other could sometimes be separately evoked. These results suggested the presence of two types of cholinergic receptors, which were distributed differently over the axo-somatic membrane complex.

Fig. 14 summarizes in schematic fashion the regions of identified cells G-L and M-LD which were sensitive to ACh, and indicates the topographic distribution of cholinoceptive sites which gave rise to Phase I (+) and Phase II (−) type responses. A similar and almost symmetric diagram can be made for cells M-R and G-R in the other half of the buccal ganglion.

The sensitivity to ACh is non-uniform over the cell body surface. The outward facing somatic membrane of the cells was insensitive to iontophoretic ACh application. Since this part of the cell membrane receives no synaptic endings (Tauc, 1960; Bullock, 1965), its insensitivity to ACh correlates well with results obtained for skeletal muscle, in which the ACh sensitivity is normally restricted to the end-plate region of the neuromuscular junction (del Castillo & Katz, 1955). Our results do differ somewhat, however, from those obtained from other, closely related, molluscan ganglion cells. In *Aplysia* (Tauc & Gerschenfeld, 1962) and snails (Stefani & Gerschenfeld, 1969), the soma is sensitive to ACh in spite of the lack of synaptic endings there. However, on the basis of the wave form of the response to ACh, the latter authors did conclude that a restricted region of the outward facing soma lacked cholinoceptive sites.

These differences in ACh sensitivity may reflect differences not only between the somatic membrane structure, i.e. variable density of ACh

receptors, in *Aplysia* and *Navanax*, but also between the inward and outward facing portions of the soma in *Navanax*.

An alternative possibility is that the outer somatic membrane is covered by non-neuronal cell-structures, which remain after the surrounding connective tissue has been cut and reflected, and that this presents a barrier to ACh diffusion (Nachmansohn, 1970). Neuroglial cells encapsulate the outer surface of all the cells, and may provide such a barrier to diffusion, while they are much more sparse in the neuropile (Bullock, 1965). The neuroglia around the large cells in *Navanax* may present more of a barrier to ACh diffusion than is the case in *Aplysia*.

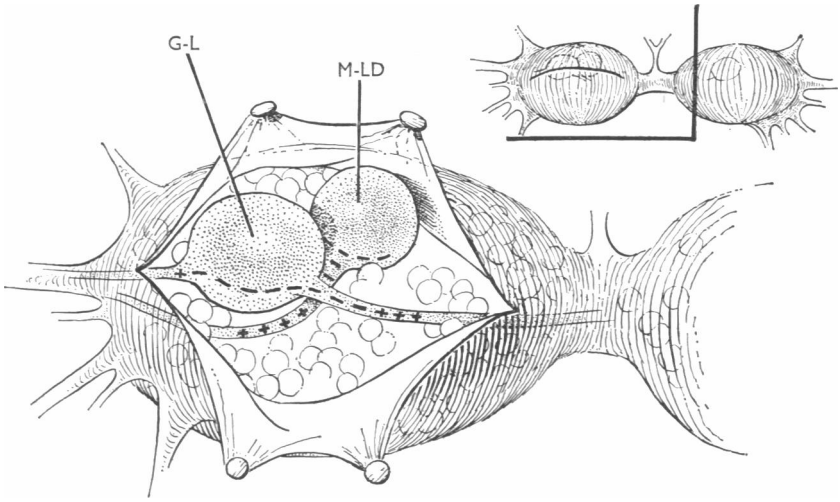

Fig. 14. Regions of ACh sensitivity illustrated on a schematic diagram of the dorsal face of the left half of the buccal ganglion. The connective tissue which normally envelops the ganglion has been cut and reflected with pins, exposing two large cells labelled G-L and M-LD. The inward facing somatic membrane responded to ACh with a potential having a reversal level near -50 mV and is donoted by $(-)$. Application of ACh to processes in the neuropile more distal to the soma produced potential changes with reversal potentials more positive than -30 mV $(+)$. Inset is drawing of entire buccal ganglion, with square indicating region dissected and enlarged.

A distinction between muscarinic and nicotinic type cholinoceptive sites was made some time ago (Dale, 1914) and supporting pharmacological evidence is plentiful for the exclusive existence of one or the other on a given structure (Goodman & Gilman, 1965). It is only recently that two types of cholinoceptive sites have been found on the same receptor unit (Curtis & Ryall, 1966; Kehoe, 1967; Wachtel & Kandel, 1967, 1971; Koketsu, 1969; Barker, Crayton & Nicoll, 1971).

The pharmacological properties of the two receptor types reported here preclude their classification as either nicotinic or muscarinic. The receptor associated with Phase I was blocked by both muscarinic and nicotinic antagonists. Moreover atropine was ineffective as an antagonist at the second receptor, which exhibited many muscarinic properties, being activated by MeCh, oxytremerine and pilocarpine. Tetraethylammonium, shown to be an effective antagonist to ACh at receptors which resisted a variety of cholinolytics (Kehoe, 1967, 1969*a*) was without effect on Phase II.

In several cases where ACh has been shown to change the membrane permeability to chloride, curare was found to be an effective antagonist (Tauc & Gerschenfeld, 1962; Wachtel & Kandel, 1967; Kehoe, 1967; Blankenship, Wachtal & Kandel, 1971). In the present case, however, D-tubocurarine was ineffective in blocking the chloride-dependent, Phase II response to ACh.

As a whole the results summarized above suggest that caution should be exercised in the interpretation of the effects of cholinolytics. The inability of curare to block transmission at a synapse where there is a permeability change to chloride, and the impotence of other classical cholinolytics may not justify the elimination of ACh as a transmitter candidate at the site in question.

In a recent monograph Eccles has summarized what he considers to be two basic principles of synaptic action (Eccles, 1969, p. 112). The first principle is derived from Dale's suggestion (Dale, 1935), that at all the terminals of a nerve cell the same substance is released. The second principle of Eccles states, '...that, at all of the synaptic terminals of a nerve cell, the transmitter substance opens just one type of ionic gate, that characterizing either excitatory or inhibitory synapses'.

It seems clear from the present study and the results of other workers that the second principle is not tenable. Kehoe (1967, 1969*b*) has convincingly shown that an identified neurone releasing the transmitter ACh opens ionic gates to both K and Cl on the same post-synaptic neurone. Wachtel & Kandel (1967, 1971) have demonstrated that another identified neurone can mediate both excitation and inhibition in follower cells and that ionic gates for Na and Cl are opened in each case (Blankenship *et al.* 1971). In the present work it was shown that ACh can open ionic gates to Na and Cl on the same neurone, thus making it possible for a single presynaptic neurone to mediate both excitation and inhibition.

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