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THE PHYSIOLOGICAL ROLE OF THREE ACETYLCHOLINE RECEPTORS IN SYNAPTIC TRANSMISSION IN *APLYSIA*

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SUMMARY

1. It is shown that a single presumably cholinergic presynaptic neurone can mediate, monosynaptically, multicomponent responses in a given cell and different responses in different cells.

2. Complex responses (whether evoked synaptically or by ACh injection) are shown to be the result of the coexistence on a given post-synaptic neurone of more than one of three cholinergic receptor types previously described. Likewise, different responses in different cells are due to the fact that different post-synaptic neurones bear different combinations of these three receptors.

3. Pharmacological analysis shows that the multicomponent nature of many of the responses is not always evident: what appears, under normal conditions, to be a single-component excitatory potential can be shown often to be a complex response consisting of superimposed e.p.s.p.s and rapid i.p.s.p.s which are sometimes, though not always, accompanied by a slow i.p.s.p.

4. Although which and how many of the three receptor types is the major factor contributing to the type of response observed, in the case of some of the synaptic potentials certain other factors were found to contribute to the final response form. First, in the large cells of the visceral ganglion, as well as in the left giant cell of the pleural ganglion, there is a marked 'electrical separation' between the region in which the synaptic currents are generated and the point of recording. This 'electrical distance' often altered the inversion potential, and sometimes the form of the responses. Secondly, in some visceral neurones, activation of the cholinergic presynaptic neurone L10 causes (either directly or indirectly) a potential

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change which cannot be accounted for in terms of the activation of cholinergic receptors. This 'non-cholinergic' response (not imitated by an ionophoretic injection of ACh) is unmasked by the blocking of all three cholinergic receptors. It contributes differentially in different cells to the total response pattern produced by L10 under normal conditions, but its contribution is often characterized by a late hyperpolarizing phase which appears to be impossible to invert. This phase has been shown, however, to be dependent upon the potassium concentration in the extracellular space surrounding the synapse.

4. It is tentatively suggested that this residual, non-cholinergic element of the synaptic response in some visceral cells be the result of the activation of an electrical synapse.

INTRODUCTION

Recent investigations of synaptic transmission in *Aplysia* neurones have revealed that, in addition to the classically described e.p.s.p.s and i.p.s.p.s, there exists a wide variety of multicomponent synaptic potentials (see for example, Kehoe, 1969*b*, 1971; Pinsker & Kandel, 1969; Wachtel & Kandel, 1971).

It has also been observed in *Aplysia* that firing of a single presynaptic neurone can cause different types of responses in different follower cells (Strumwasser, 1962; Kandel, Frazier, Waziri & Coggeshall, 1967; Gardner, 1971).

The first purpose of this paper is to show that multicomponent responses in a given cell and different responses in different cells can all be mediated monosynaptically by the same presynaptic neurone. A similar effort has been made using identifiable cells in the visceral ganglion, and latency as a criterion of monosynaptic contact (Kandel *et al.* 1967; Wachtel & Kandel, 1971). A more complete demonstration of monosynaptic connexions has been sought here by analysing the effects of modification in transmitter release from an identifiable presumably cholinergic neurone, which provokes a two-component inhibition (one rapid element, one slow) in the medial cells of the pleural ganglion, and a simple excitatory response in the anterior cells.

It was shown in the second paper of this series (Kehoe, 1972*b*) that the three types of potential change (rapid e.p.s.p., rapid i.p.s.p., slow i.p.s.p.) observed in the pleural ganglion cells are mediated by three pharmacologically distinct receptor types. Thus, the two-component inhibitory response in the medial cells is due to the activation of two different receptor types coexisting on the same post-synaptic cell, whereas the rapid e.p.s.p. in the anterior cells is mediated by still a third receptor type.

The final purpose of this paper was to perform a pharmacological analysis

of the complex responses observed in the visceral ganglion to see if, as in the pleural ganglion, the type of response given by a cell is simply a function of which and how many of the three cholinergic receptor types it bears. Brief accounts of some of these experiments have been published previously (Kehoe, 1969*b*; Kehoe & Ascher, 1970).

METHODS

With only a few exceptions, the methods used were the same as those described in the first paper of this series (Kehoe, 1972*a*). In certain experiments, however, an interbarrel injection (see Eccles, Eccles & Ito, 1964) of TEA sulphate was made in presynaptic neurone I. One of the two barrels of the micro-electrode placed in the presynaptic neurone was filled with TEA Cl or TEA Br (10^{-2}–10^{-1} M), and the other was filled with the standard solution of 0·6 M-K_2SO_4.

When the ganglion was exposed to calcium-free sea water, the controls were performed in artificial sea water (composition, in m-mole/l.: Cl^- = 592; Na^+ = 468; HCO_3^- = 6; Mg^{2+} = 50; K^+ = 10; Ca^{2+} = 10). In the calcium-free solution, the magnesium concentration was augmented to 60 m-mole/l., and EGTA (10^{-3} M) was added.

As indicated in the text, one series of experiments required the use of *Aplysia fasciata* and *A. rosea*, in addition to *A. californica* used in all other investigations reported here. Furthermore, as indicated when appropriate, some data were gathered from the visceral rather than from the pleural ganglion. The identification of visceral neurones was made on the basis of data presented by Frazier, Kandel, Kupfermann, Waziri & Coggeshall (1967), whereas cells in the pleural ganglion were identified according to data presented in the second paper of this series (Kehoe, 1972*b*).

In the experiments in which double barrelled micro-electrodes were inserted in both the presynaptic cholinergic neurone L 10 of the visceral ganglion and in one of the follower cells, the ganglion was placed ventral side up, and the connective tissue of that side was dissected away. It was often necessary to perform a rather deep and thorough dissection to identify and reach, with the double barrelled electrode, cells which are more readily visible from the dorsal surface (e.g. L 1–L 6, R 15).

All of the drugs used in experiments presented in this paper are included in the list given in the second article of this series (Kehoe, 1972*b*).

RESULTS

Description of the two-component inhibitory response

When presynaptic neurone I is activated, a two-component response can be recorded in the so-called medial cells of the pleural ganglion (see Kehoe, 1972*a* for nomenclature). This two-component response is composed of a rapid inhibitory potential (rapid i.p.s.p.) which is superimposed upon and outlasted by a slowly developing inhibition (slow i.p.s.p.). At resting level, both phases are hyperpolarizing (see Fig. 1*A*). For a description of this type of response as a function of membrane potential see the legend of Fig. 8*A*.

When the presynaptic neurone is fired repetitively (at approximately 25/sec) the latency between the peak of the presynaptic spike and the onset of the rapid i.p.s.p. is constant in a given preparation. Fig. 1*B* gives an example taken when the membrane potential is pre-set at −80 mV so that

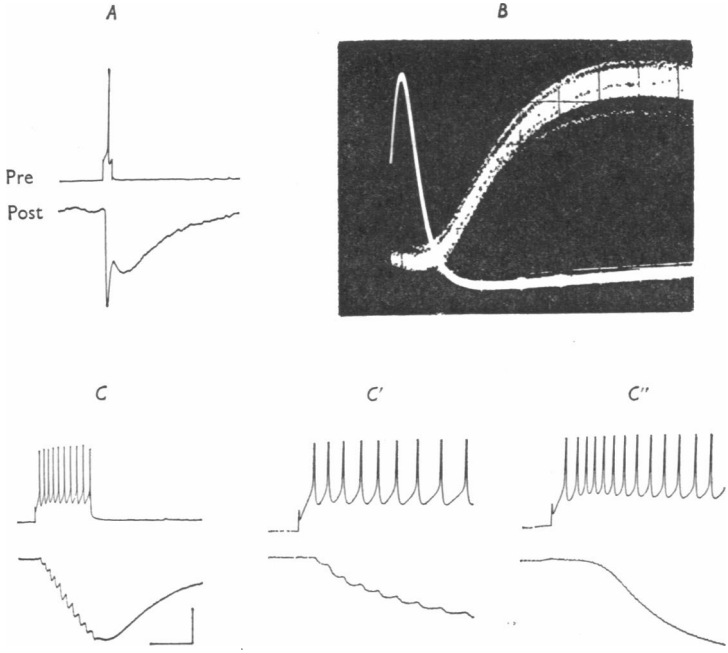

Fig. 1. *A*, two-component inhibitory response of medial pleural cell at resting level (Post) to a single presynaptic spike (Pre). *B*, rapid i.p.s.p. of medial pleural cell in response to high frequency firing of the presynaptic neurone I. The medial cell was pre-set at −80 mV, at which level the rapid i.p.s.p. is inverted and the slow i.p.s.p. is in 'equilibrium'. *C*, typical response of medial pleural neurone to repeated firing of presynaptic neurone I. A rapid i.p.s.p. is associated with each presynaptic spike, whereas the slow i.p.s.p. is only evident with repeated firing, and is seen as a summated slow wave. *C'*. Same as *C*, but with high speed recording. *C''*. Same as *C'*, but after the ganglion has been curarized, blocking the rapid i.p.s.p. Note that the slow i.p.s.p. appears gradually, and only after the presynaptic neurone has been fired a few times. Curare often causes an increased excitability of the presynaptic neurone, which causes an increased firing rate of the presynaptic neurone in response to the same depolarizing pulse. Calibration, *A*: 2 sec, 5 mV; *B*: 10 msec, 10 mV (presynaptic); 5 mV (post-synaptic); *C*: 200 msec, 5 mV (post-synaptic).

the rapid i.p.s.p. is inverted and the slow i.p.s.p. is in equilibrium, thus not interfering with the repetitive measurement of the latency of the rapid potential. In this experiment, the latency was approximately 6 msec. The values obtained in a number of preparations ranged from 4·5 to 7 msec.

A number of factors impede the exact measurement of the latency of the slow i.p.s.p. First, the development of the slow i.p.s.p. is so gradual that it is difficult to define the beginning of the response, and the response is so long lasting that testing under high frequency firing (to determine its constancy) is impossible. The approximate latency of the slow i.p.s.p. (measured after the rapid i.p.s.p. has been eliminated pharmacologically) is 20 times that of the latency to the rapid i.p.s.p. Another frequent characteristic of the response also impedes a description of the latency of the slow i.p.s.p.; that is, in many preparations no measurable slow phase occurs in response to a single presynaptic action potential. In Fig. 1*C* is an example of a case where the second phase develops only with repeated firing. In Fig. 1*C'*, *C''*, high speed recordings of the response before and after elimination of the rapid i.p.s.p. by tubocurarine (see Kehoe, 1972*b*) permits a comparison of the relationship of the rapid and slow components, respectively, to the presynaptic action potential. As can be seen in Fig. 1*C''*, the slow i.p.s.p. in this experiment was not measurable until a number of presynaptic spikes had occurred in succession. This record should be compared with that of Fig. 1*A*, in which a single spike elicited both a rapid and slow i.p.s.p. Since there is a marked post-tetanic potentiation of the two-component inhibition, even in preparations such as that shown in Fig. 1*C*, an additional spike a few seconds following the end of the burst would cause a clearly two-component response such as that seen in Fig. 1*A*.

Effects of modification of transmitter release on the two-component inhibitory response

Effects of calcium deprivation. Exposure to calcium-free sea water induced a progressive reduction of the amplitude of both components of the inhibitory response. This can be seen in Fig. 2*A–F*, which shows response amplitude as a function of the time in calcium-free solution (*A* = 0 min; *F* = 30 min). Each pair of traces consists of the recording of the spikes from the presynaptic neurone (Pre) and the response these spikes caused in the post-synaptic neurone (Post). The initial rapid i.p.s.p. of each test (see arrow in lower trace of the pair in Fig. 2*A*) was used as a measure of the rapid i.p.s.p. amplitude. Since the slow i.p.s.p. resulting from a single presynaptic spike is often not measurable, even in normal calcium, it was necessary to use a summated slow wave as the indicator of the slow i.p.s.p. amplitude (see dash on lower trace in Fig. 2*A*).

A plot of the values of the amplitudes of the rapid and slow i.p.s.p.s measured in records *A* to *F* is presented in the lower half of Fig. 2. Statistical evaluation of these data confirms that the amplitudes of the two components decrease together ($r = 0{\cdot}89$; null hypothesis rejected at the 0·005 level of confidence).

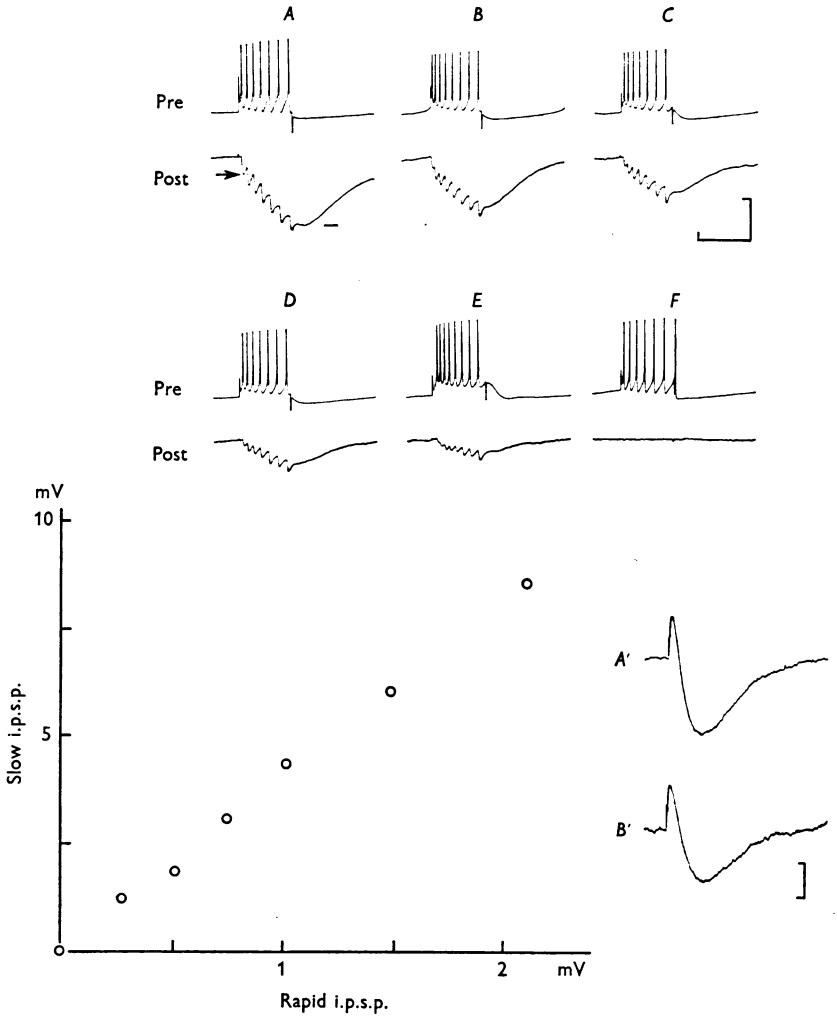

Fig. 2. Effects of a 30 min exposure to calcium-free sea water on two-component inhibition of medial pleural cells. With continued exposure, both components of the post-synaptic response (see lower trace, Post, of each pair *A–F*) decrease in amplitude, and are eventually eliminated completely (see *F*, 30 min exposure). Calibration: 1 sec, 5 mV. The amplitude of the initial i.p.s.p. of a series (see arrow in Post, *A*) is plotted against the amplitude of the summated slow wave (see dash in Post, *A*) in the lower half of the Figure. To the right of that plot is a record of the ACh potential, measured at -70 mV, in normal sea water (*A'*) and after a 45 min exposure to calcium-free sea water (*B'*). Calibration: 10 sec, 5 mV.

As can be seen in Fig. 2*A'*, *B'*, a 45 min exposure of the ganglion to calcium-free sea water does not significantly alter the response to an iontophoretic injection of ACh. Nor could a change in the action potential of the presynaptic cell in the absence of calcium be detected (although the pen recordings taken would not reflect certain changes that could perhaps be seen in a more analytical study). Thus it appears that the progressive reduction of the two components of the post-synaptic response is indeed due to a selective effect on the transmitter release mechanism.

Effects of TEA injection in the presynaptic neurone. Calcium-free solutions act on all cells of the ganglion and thus cause depression of transmitter release from all neurones. Intracellular injection of TEA, on the contrary,

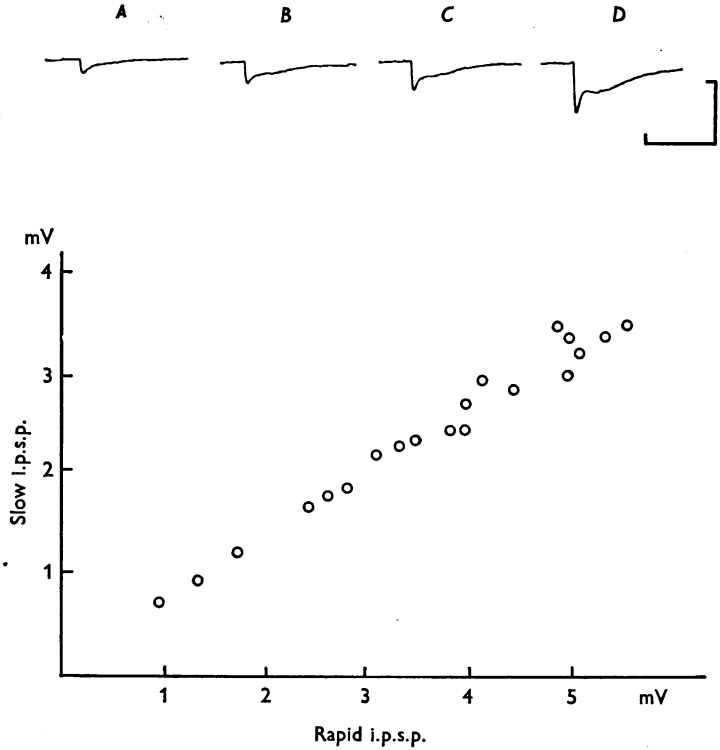

Fig. 3. Effects of intracellular injection of TEA in the presynaptic neurone I on the amplitude of both components of the two-component inhibitory response in medial pleural cells. *A–D* show sample records of responses as a function of increasing duration of TEA injection. When the amplitude of the two components of the twenty responses periodically recorded in one experiment are plotted against each other, it can be seen that the changes in amplitude of the two components are highly correlated. Calibration: 1 sec, 5 mV.

provides a means of varying the release from a single neurone. TEA interferes with potassium permeability and by this means increases the duration of the action potential in a variety of preparations (see Hille, 1970, for references). A prolongation of the action potential, in turn, causes an increase in transmitter release (Katz & Miledi, 1967; Kusano, Livengood & Werman, 1967). Since TEA has been found to have similar effects on the action potential of *Aplysia* neurones (Kehoe, 1969*a*, 1972*a*) this ion was injected into the presynaptic neurone to prolong its action potential and thereby increase transmitter release from this neurone.

Fig. 3 gives four examples of the response to a single presynaptic spike with increasing (*A* to *D*) intracellular TEA in the presynaptic neurone. As the concentration of TEA increased with continued injection, both components of the response increased. Moreover, this increase was progressive and a change in amplitude of one component was accompanied by a change in amplitude of the other. In the lower half of Fig. 3, the values of the amplitudes of the rapid and slow i.p.s.p.s of the responses measured in this experiment are plotted one against the other, and a statistical evaluation of the relationship between them reveals that they are very highly correlated ($r = 0{\cdot}98$, null hypothesis rejected at the 0·001 level of confidence).

Effects of modification of transmitter release on different response types in different post-synaptic cells

As was shown above, activation of presynaptic neurone I causes a two-component inhibitory response in the medial cells of the pleural ganglion.

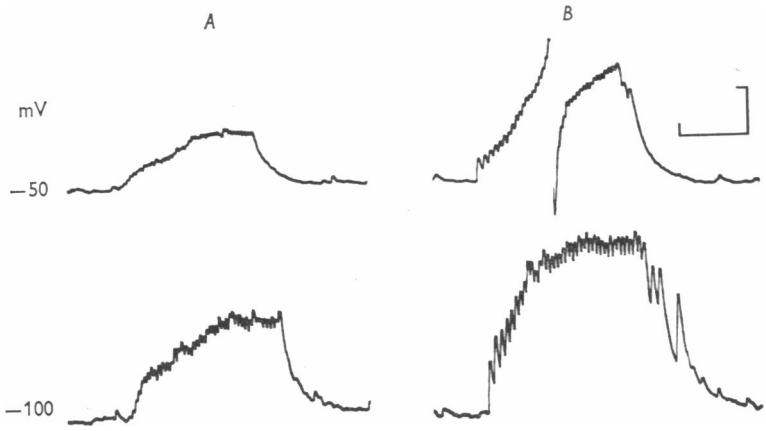

Fig. 4. Response of anterior pleural neurones to repetitive firing of presynaptic neurone I before (*A*) and following (*B*) injection of TEA in the presynaptic neurone. Calibration: 2 sec, 5 mV.

In contrast, this same neurone causes a single component excitatory response (e.p.s.p.) in the anterior cells of this same ganglion. The e.p.s.p.s provoked in one of the anterior cells by a series of presynaptic action potentials are shown in Fig. 4*A*. The record from the presynaptic neurone is not shown, but can be readily imagined since each e.p.s.p. is associated with a single presynaptic spike. As can be seen in Fig. 4*B*, an intracellular injection of TEA in the presynaptic neurone causes a marked increase in the e.p.s.p.s it produces in these cells. This increase is progressive and directly related to the duration of the injection of TEA. When simultaneous recordings were made from a medial and an anterior neurone, as well as from the presynaptic neurone exposed to TEA injection, the increase in the e.p.s.p. amplitude in the anterior neurone coincided with the increase in both components of the two-component inhibition in the medial cell.

Pharmacological analysis of a variety of cholinergic responses of visceral ganglion cells

It was shown in the second article of this series (Kehoe, 1972*b*) that three different types of cholinergic receptors can be distinguished pharmacologically in *Aplysia* neurones. The receptor mediating excitation can be blocked selectively by hexamethonium, a drug which has no effect on either of the receptors mediating the inhibitory responses. The rapid phase of the inhibitory response can be blocked by tubocurarine, which has no effect on the receptor mediating the slow hyperpolarization. The slow i.p.s.p., on the other hand, can be selectively blocked by methylxylocholine (see Kehoe, 1972*b* for further details). Thus, the excitatory response observed in the anterior cells can be separated pharmacologically from the two inhibitory responses of the medial cells, which in turn can be independently eliminated. Consequently, it can be concluded that the different responses of the different pleural neurones, as well as the separate components of the polyphasic inhibitory response, are the result of the monosynaptic, cholinergic activation by presynaptic neurone I of three different receptor types, each mediating one of the three different responses (rapid e.p.s.p., rapid i.p.s.p., slow i.p.s.p.).

It will be shown here that the variety of the cholinergic synaptic potentials observed in the *visceral* ganglion in different cells in response to activation of the cholinergic neurone L 10 is likewise due to various combinations, on the different post-synaptic cells, of the three cholinergic receptor types. The complexity of the response is sometimes immediately evident; sometimes only after pharmacological treatment.

Pharmacological break-down of a 'simple' response into excitatory and inhibitory components. Fig. 5*A* shows the response of an unidentified cell found on the ventral surface of the visceral ganglion to stimulation of L 10.

Although the recordings of the presynaptic action potentials are not included in the figure, the frequency of presynaptic firing is reflected by the frequency of the rapid post-synaptic potentials. At first sight, the response appears to be a simple, excitatory synaptic potential. However, when the ganglion is exposed to hexamethonium, which selectively blocks the excitatory response to ACh or the synaptically activated 'e.p.s.p.', an underlying i.p.s.p., insensitive to hexamethonium, is revealed (Fig. 5*B*). Curare eliminates the i.p.s.p. (Fig. 5*C*), but leaves a slow depolarizing wave that is not imitated by ACh ionophoretic injection, nor blocked by other ACh antagonists (see below).

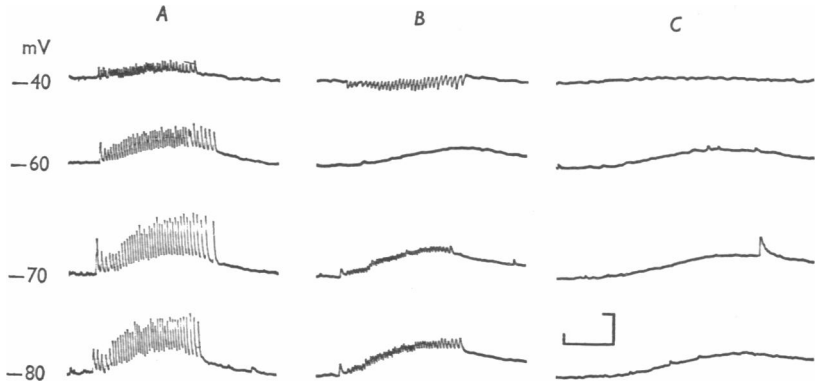

Fig. 5. Pharmacological separation of two cholinergic receptors on a single unidentified neurone of the visceral ganglion. *A*, response, as a function of membrane potential, to repeated firing of presynaptic cholinergic neurone L 10. Each 'e.p.s.p.' is correlated with a single presynaptic spike (not shown). *B*, response of same neurone to L 10 stimulation following addition of hexamethonium 10^{-3} M to bathing medium. This drug eliminates the excitatory element of the response (which was depolarizing at all levels in *A*), and reveals a rapid i.p.s.p. of similar time course which inverts at −60 mV. *C*, following the addition of tubocurarine the i.p.s.p. is eliminated, leaving only a very slowly developing depolarizing response to presynaptic stimulation. This slow depolarization was unaffected by all cholinolytics tested. Calibration: 2 sec, 5 mV.

Pharmacological break-down of a complex response into three components. An even more complex response, shown in Fig. 6, was recorded from another unidentified cell found on the ventral surface of the visceral ganglion in response to stimulation of L 10. This response (see Fig. 6*A*, −45 mV) appears to be predominantly excitatory, but an underlying slow hyperpolarization is revealed by the slow drift of the base line as the presynaptic stimulation is repeated. This slow hyperpolarization is most prominent at the termination of the presynaptic firing (identified by the final rapid e.p.s.p.). The dashed line indicates what the normal membrane

potential would be at the end of presynaptic firing if there was no underlying slow i.p.s.p. present. Note that this slow hyperpolarization, like the slow i.p.s.p. in the medial pleural cells, inverts at −80 mV. The two components (e.p.s.p. and slow i.p.s.p.) that are apparent in the normal preparation are in fact accompanied by a third component (a rapid i.p.s.p.) that is revealed only after the e.p.s.p. is blocked by hexamethonium. In

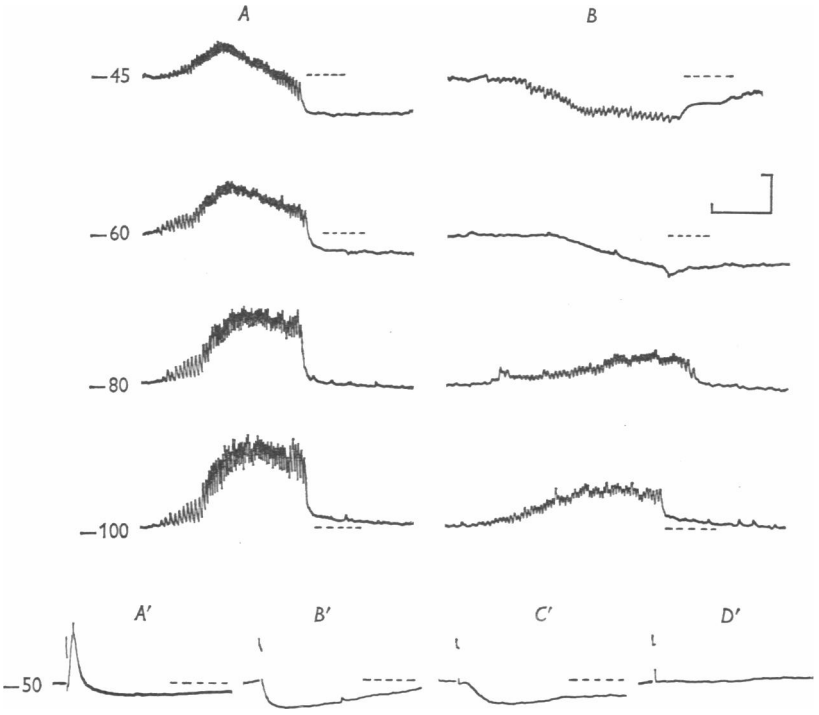

Fig. 6. Pharmacological separation of three cholinergic receptors on a single unidentified neurone of the visceral ganglion. *A*, response, as a function of post-synaptic membrane potential, of an unidentified neurone to repetitive stimulation of presynaptic cholinergic neurone L10. Each 'e.p.s.p.' is correlated with a single presynaptic spike (not shown). *B*, response of same neurone to L10 stimulation following addition of hexamethonium (10^{-3} M) to bathing solution. This drug eliminates the excitatory element of the response, unmasking a simultaneously activated two-component inhibitory response (compare *A*, *B* at −45 mV). The slow i.p.s.p. can be identified by the slow hyperpolarizing drift which counteracts the excitatory response in *A* and amplifies the hyperpolarization caused by the rapid i.p.s.p. in *B* (see −45 mV). Note the slow return of the base line at the end of presynaptic firing caused by this slow i.p.s.p., except at −80 mV (its potential of inversion). Calibration: 2 sec, 5 mV. *A′*–*D′*, response of same cell to ACh injection in normal conditions (*A′*), under hexamethonium (*B′*); under tubocurarine (*C′*); under tubocurarine + methylxylocholine (*D′*). See text for interpretation. Calibration: 8 sec, 5 mV.

column *B* of Fig. 6 (see −45 mV in particular) this rapid i.p.s.p. is seen superimposed upon the slow i.p.s.p. already evident in the untreated preparation. The response in the presence of hexamethonium is thus of the same type as that observed in the medial pleural cells in the normal preparation, consisting of a rapid i.p.s.p. superimposed upon a slowly-developing, long-lasting i.p.s.p. (see, for example, Fig. 8*A* in this paper). As is the case for the response in the medial pleural cells, the rapid component in this visceral cell can be blocked by curare, whereas the slow can be eliminated by methylxylocholine (see examples of such selective blockades in Figs. 2 and 5 of the second article of this series; Kehoe, 1972*b*).

The response of ACh in the cell studied in Fig. 6 could also be broken down into three components, as can be seen in the last line of that figure (*A'*–*D'*). The response in this cell, whether elicited synaptically or by ACh injection, is thus due to *the activation on the same post-synaptic neurone of all three cholinergic receptor types previously defined.*

The ACh response in *A'*, obtained in the untreated preparation, appears to consist of a depolarizing response superimposed upon a slow hyperpolarization – revealed by the fact that the post-depolarization level is hyperpolarized in comparison with the pre-response membrane potential of −50 mV. When bathed in hexamethonium (Fig. 6*B'*) the excitatory phase is blocked, leaving a hyperpolarizing response. That the remaining hyperpolarization is the manifestion of a two-component hyperpolarizing response can be seen in this series of records by the shift in latency that occurs when curare is added to the bath (compare Fig. 6*B'* and 6*C'*). The shift in latency represents the block of the rapid component of the ACh potential which, like the rapid i.p.s.p. of the synaptic response, inverts at −60 mV (see Fig. 3 at −45 mV, Kehoe, 1972*b*, for a comparable record from the medial cells). The remaining response is a longer-latency, longer lasting hyperpolarization which, like the slow i.p.s.p., inverts at −80 mV and is blocked by methylxylocholine.

Species differences in cholinergic responses in Aplysia neurones. The response of the medial cells of the pleural ganglion to ACh ionophoretic injection and cholinergic synaptic stimulation has been observed to differ in three species of *Aplysia: A. californica*, *A. fasciata*, and *A. rosea*. The slow component of the ACh response, which is very marked in *A. californica* (Fig. 7*A*) is only barely noticeable in *A. fasciata* (Fig. 7*B*) and could not be detected at all in *A. rosea* (Fig. 7*C*). There were similar differences in the response to synaptic stimulation, as can be seen in the records presented at the bottom of Fig. 7. Trace *A'* is the synaptic response to mechanical stimulation of the tentacles (using the isolated head preparation, see Bruner & Tauc, 1966) recorded from *A. californica*; the same response, recorded in *A. fasciata* (*B'*) shows practically no slow phase.

The interpretation of these differences remains doubtful. There has been no control for season, age, differences in living conditions, or for

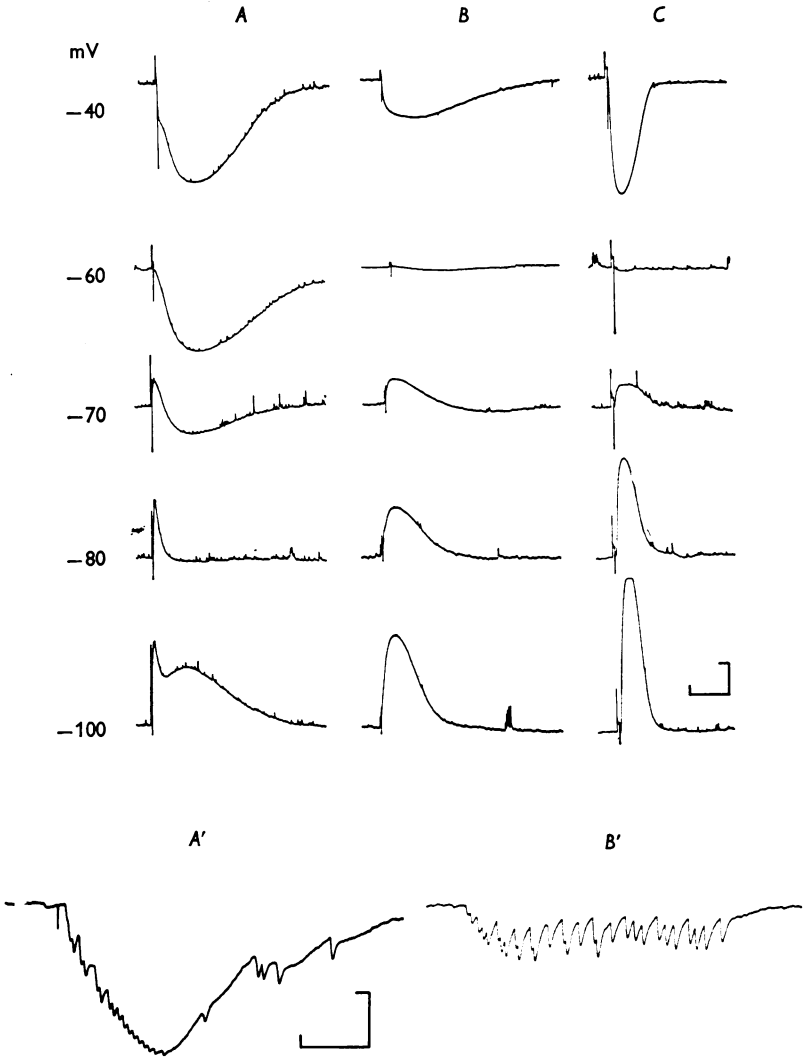

Fig. 7. *A*, *B*, *C*, responses of medial pleural cells to ionophoretic injection of ACh in *Aplysia californica* (*A*), *A. fasciata* (*B*), and *A. rosea* (*C*). The slow component which inverts at −80 mV is prominent in (*A*), barely detectable in (*B*), and absent in (*C*). Calibration: 10 sec, 5 mV. That the lack of a slow component in the ACh response in *A. fasciata* is not merely an artifact of iontophoretic injection procedures is shown by a similar difference in the synaptic responses of *A. californica* (*A′*) and *A. fasciata* (*B′*). Both of these synaptic potentials (recorded at approximately −45 mV) represent responses to stimulation of the tentacles (using the isolated head preparation) which has been shown to activate presynaptic neurone I, which in turn causes the inhibitory response in the medial pleural cells. Calibration: 2 sec, 5 mV.

size of the animals, all of which are confounded with the change in species. The practical result of this finding, however, was the exclusive use of *A. californica* for the experiments reported in these papers.

Effect of distance between the point of recording and the point of origin of synaptic currents

The two-component synaptic inhibition observed in the medial pleural cells is also observed in the giant cell (Hughes & Tauc, 1963) of that same ganglion. This response, however, often presents some unique characteristics. First, in order to obtain a post-synaptic potential change of an amplitude equivalent to that observed in the medial cells, it is necessary to fire the presynaptic neurone more rapidly and for a more prolonged period. This can be seen in Fig. 8, where the recording from the giant cell (Fig. 8*B*) was taken at approximately one quarter of the speed of that of the medial cell (Fig. 8*A*), and where the length of the line over each post-synaptic recording indicates the duration of presynaptic firing. Secondly, the rapid i.p.s.p. in the giant cell is always rounded in comparison to the corresponding potential of the medial cells, which is much more abrupt. Thus, it is often difficult to distinguish the rapid i.p.s.p. of the giant cell when, for example, at -45 mV, it is of the same polarity as the slow response. This is particularly true for records taken at a relatively slow speed (as in Fig. 8*B*). Thus, the rapid i.p.s.p. in the giant cell is more evident in the records taken when the cell is hyperpolarized beyond the inversion level

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Legend to Fig. 8.

Response of a medial pleural neurone (*A*) and of the left giant cell (*B*) to firing of presynaptic neurone I (indicated by line above each recorded response). *A*, response of medial pleural neurone to approximately 15 presynaptic spikes fired at 6/sec for 3 min. At resting level (-45 mV) both components of the response are clearly visible. The rapid i.p.s.p.s (one corresponding to each presynaptic spike) are superimposed upon the summated slow i.p.s.p.s. The rapid response is in 'equilibrium' at -60 mV. At this membrane potential, the slow i.p.s.p. is still hyperpolarizing, and thus causes an inversion of the rapid i.p.s.p.s triggered by later spikes in the series (see arrow). The slow i.p.s.p. is in 'equilibrium' at -80 mV, and clearly inverted (note slow return to base line at the end of presynaptic firing) in the record taken at -100 mV. *B*, in order to obtain a response of equivalent amplitude in the left giant cell, it is usually necessary to fire the presynaptic neurone approximately twice as fast for twice as long. The response of that cell to a 10/sec firing rate for a 6 sec period is shown in (*B*). Note that the slow component in the giant cell did not invert until the soma was hyperpolarized beyond -100 mV. Calibration, *A*: 1·5 sec, 5 mV; *B*: 6 sec, 5 mV. Note that the record in *B* is taken at approximately one quarter of the speed of the record in *A*.

of the rapid phase (see -70 mV, at which level a depolarizing component can be seen superimposed upon the slow wave hyperpolarization). A third significant difference between the records from the giant cell and those of the medial cells is that in the giant cell the slow i.p.s.p. does not usually

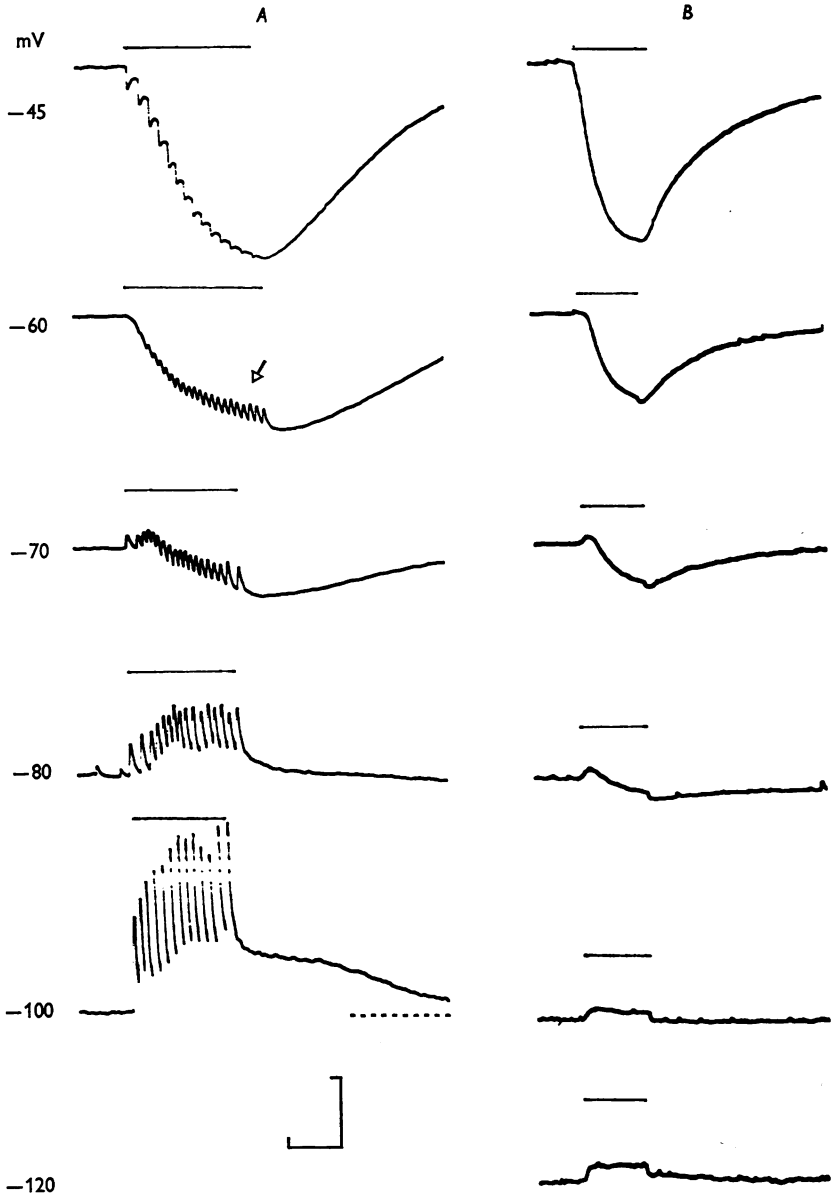

Fig. 8. For legend see opposite page.

invert until the soma is brought to approximately −100 mV (rather than −80 mV, the potential of inversion of the slow i.p.s.p. in the medial cells). All these deviations in the response characteristics of the giant cell two-component synaptic inhibition are assumed to be the result of a greater 'electrical' distance between the point of recording (soma) and the point of origin of the synaptic currents.

Similar distortions of the two-component synaptic inhibitory response to stimulation of L 10 can be seen in some of the large 'follower cells' of the visceral ganglion. For example, in many of the cells of the group L 1–L 6 (see Kandel *et al.* 1967, for nomenclature) the inversion potential of the slow component of the synaptic response is very high, and sometimes the response cannot be inverted even with a somatic potential of −135 mV. The somatic response of the same cells to an iontophoretic injection of ACh, on the other hand, is identical to that of the medial pleural neurones: that is, the slow phase inverts at −80 mV. It could be anticipated, *a priori*, that an ionophoretic injection of ACh on the soma might not reach all receptor types involved at the synaptic region, and that the response resisting inversion might be the result of the activation of another cholinergic receptor type found exclusively in the synaptic region. However, as has been shown by Kunze & Brown (1971), the response of these visceral cells to a bath application of ACh inverts at approximately −80 mV (when the early response is blocked by curare). These facts suggest that the 'anomalous' inversion potential of the synaptic response is undoubtedly not due to the activation of still another cholinergic receptor type, but rather to a distortion of the pleural cell prototypic slow wave by other factors. One of these factors is undoubtedly distance between the point of recording and point of origin of synaptic currents, as it appears to be in the giant cell of the pleural ganglion. That such a 'distance' factor could play a role in these visceral cells is further suggested by the data presented in Fig. 9. When a double barrelled micro-electrode was introduced into both the soma and the presumed synaptic region (Kandel *et al.* 1967) of one of these cells, hyperpolarizing the soma to approximately −80 mV polarized the axonal membrane of the synaptic region to only approximately −60 mV. Conversely, polarization of the axonic region to approximately −80 mV brought the soma to only −60 mV.

Although the existence of 'distant junctions' likely accounts in part for the anomalous inversion potentials obtained for the slow i.p.s.p.s in certain visceral cells, as it most probably does for that of the giant cell of the pleural ganglion, in certain of the visceral ganglion cells there is an additional potential which occurs coincidentally with the slow i.p.s.p.s of the cholinergic response. This presumably non-cholinergic potential (see below) undoubtedly contributes to the difficulty encountered in some of

the L1–L6 cells in inverting the slow hyperpolarizing component of the synaptic response.

Non-cholinergic elements in some synaptic potentials

In certain of the visceral neurones with which L10 makes synaptic contact, some components of the response to L10 activation appear to be non-cholinergic. These potential changes, which take slightly different forms

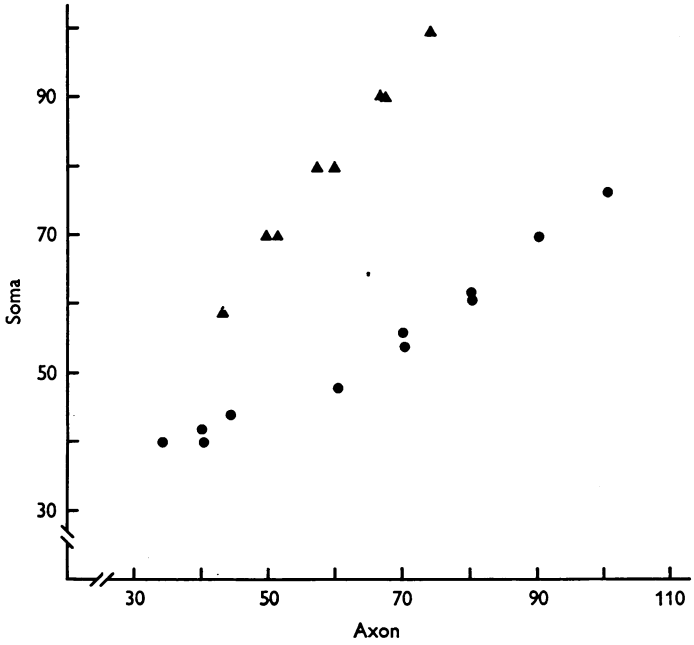

Fig. 9. Differences between axonic and somatic membrane potentials. Data from a visceral cell of group L1–L6 in which double-barrelled microelectrodes had been inserted both in the soma and in the presumed synaptic region of the axon (see Kandel *et al.* 1967). Somatic and axonic membrane potentials were compared when current was passed either through one barrel of the somatic electrode (triangles) or through one barrel of the axonic electrode (circles). Note that if there was no decrement with distance in the potential changes caused by such polarization, all points would fall on the 45° angle.

in different post-synaptic cells, share the following characteristics: they are not imitated by an iontophoretic injection of ACh, and they are not blocked by any one or any combination of the drugs used to block the three cholinergic receptors of the medial and anterior pleural cells. On the other hand, all components of the response to iontophoretic injection of ACh, as well as all synaptic potentials which are imitated by ACh in a given

cell, *can* be eliminated by these drugs. Thus, it is concluded that these responses which are not imitated by ACh injection and which resist pharmacological block are non-cholinergic.

Slow depolarizing response. In the visceral cell studied in Fig. 5, which responded with a cholinergic e.p.s.p. and rapid i.p.s.p., a drug-resistant slow depolarizing wave is clearly seen after the e.p.s.p. and i.p.s.p. have been blocked by hexamethonium and curare (see Fig. 5*C*). This slow depolarization remains even when methylxylocholine is added to the hexamethonium and curare solution.

Biphasic, predominantly excitatory response. In Fig. 10, a non-cholinergic response is shown for a cell responding with a two-component cholinergic inhibition when presynaptic neurone L 10 is stimulated. In the untreated preparation (see Fig. 10*A*) the predominant response is the two-component inhibition. The duration of presynaptic firing is indicated by the line above each post-synaptic recording. Curare, which blocks the rapid i.p.s.p. (often difficult to discriminate in these large visceral cells, as was shown to be the case for the giant cell of the pleural ganglion) reveals a depolarizing potential which is often difficult to distinguish from a partially blocked inverted i.p.s.p., and which is counteracted by the still unblocked potassium-dependent (Kehoe, 1972*a*; Kehoe & Ascher, 1970) slow i.p.s.p. The depolarizing element, which is most evident at hyperpolarizing levels where the slow i.p.s.p. interferes less with its expression (see -75 mV, Fig. 10*B*), is unmasked following a blockade of the slow i.p.s.p. with methylxylocholine (Fig. 10*C*). This remaining 'non-cholinergic' depolarizing response is sufficiently strong to fire the post-synaptic cell (see Fig. 10*C*, -55 mV). ACh injection on these cells produces a two-component inhibitory response similar to that observed in the medial cells of the pleural ganglion (see Fig. 1*A*, Kehoe, 1972*a*). Both elements of the ACh response are eliminated in the combined presence of tubocurarine and methylxylocholine.

In certain of the cells of group L 1–L 6 the response which resists the ACh antagonists is predominantly hyperpolarizing, and is very difficult to invert. Since it is superimposed upon the cholinergic late i.p.s.p., it is undoubtedly partially responsible for the difficulty encountered in inverting the late response in these cells.

Biphasic, predominantly inhibitory response. Another cell that shows a predominantly inhibitory type of 'drug-resistant' response to L 10 activation is R 15 (see Frazier *et al.* 1967, for nomenclature). The depolarizing response of this cell to ACh injection can be completely blocked by hexamethonium or curare (as are all cholinergic excitatory responses thus far studied in these cells) (see Fig. 11*A'*, *B'*). The most prominent component of the response to L 10 activation in the untreated preparation (a rapid e.p.s.p.) is also blocked by hexamethonium (see Fig. 11*A*, *B*). However,

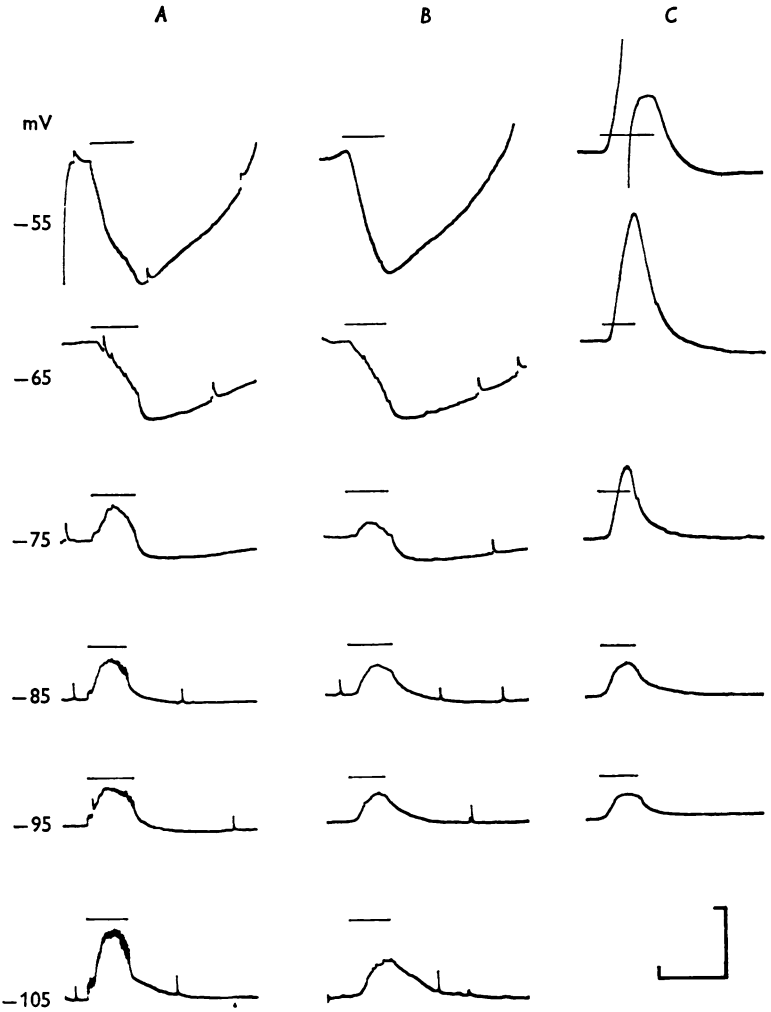

Fig. 10. Response of one of the visceral ganglion cells of group L 1–L 6 to repetitive firing (approximately 15/sec) of presynaptic cholinergic neurone L 10. Duration of presynaptic firing indicated by line above each post-synaptic record. *A*, response in normal sea water as a function of post-synaptic membrane potential. The response consists of a rapid i.p.s.p. which inverts at approximately −65 mV, and a slower i.p.s.p. which inverts between −80 and −85 mV. *B*, tubocurarine has eliminated the rapid i.p.s.p., but there still remains a rapid element superimposed upon the slow inhibitory wave. *C*, the elimination of the slow i.p.s.p. by methy-xylocholine thoroughly unmasks the depolarizing element of the 'non-cholinergic' response to L 10 stimulation, which was counteracting the slow i.p.s.p. in *B*. In this cell, this drug-resistant response is predominantly depolarizing, though a weak hyperpolarizing phase can be detected following the excitatory wave (see −65 mV). Calibration: 8 sec, 5 mV.

the pharmacological block of this cholinergic element unmasks a marked biphasic response of which the hyperpolarizing element is dominant. This biphasic response in R15 persists in the presence of hexamethonium,

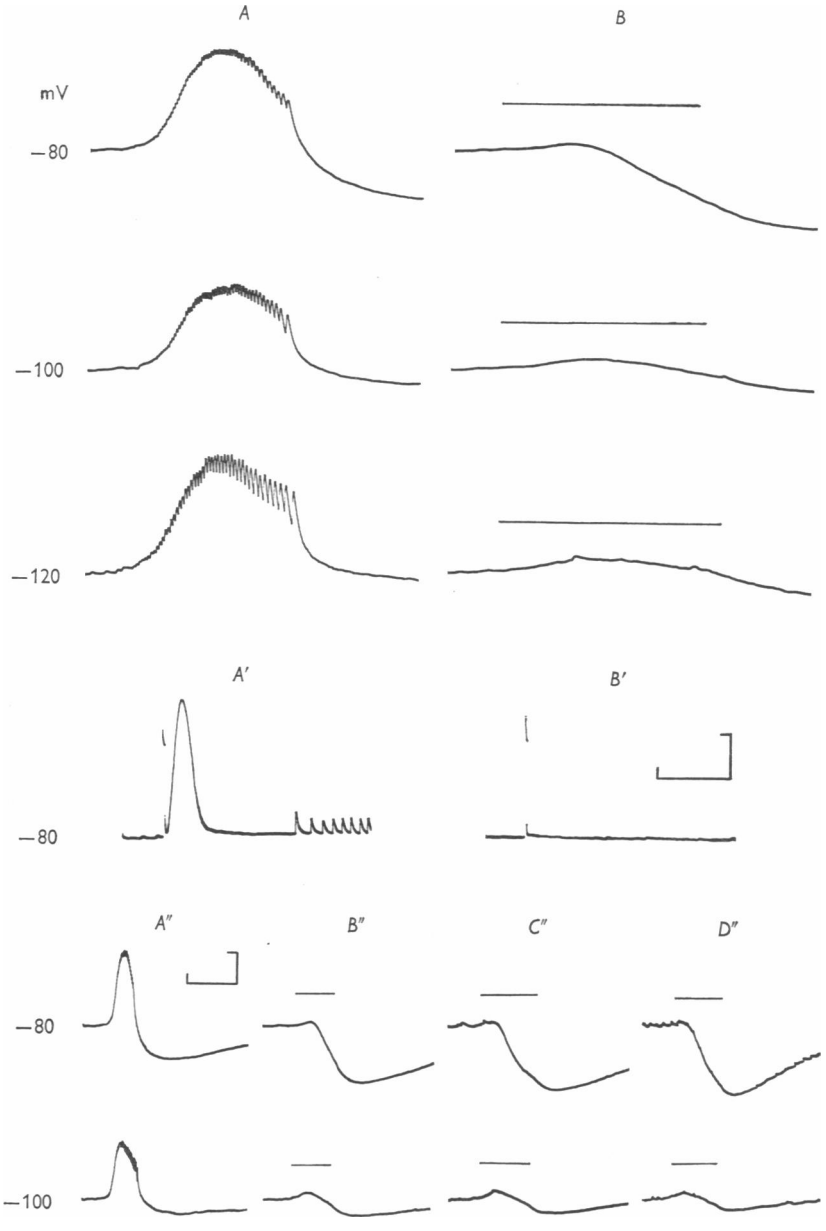

Fig. 11. For legend see opposite page.

tubocurarine, and tubocurarine plus methylxylocholine, as can be seen in records B'', C'' and D'', respectively.

The possibility that the ACh, iontophoretically applied on the soma, does not reach certain types of receptors that may exist only on more distant parts of the cell membrane must once again be considered. Such specific localization is clearly the case for dopamine receptors (Ascher, 1972) as well as for certain types of ACh receptors in another mollusc, *Navanax* (Levitan & Tauc, 1972). However, when ACh is introduced into the bathing medium while a record is taken from R 15, the only potential change observed is a depolarizing potential. And when the excitatory receptors are blocked by hexamethonium, there is no response to either bath applied or injected ACh.

DISCUSSION

A single presynaptic neurone can mediate a multicomponent response. One of the purposes of this paper was to show that a single neurone can mediate a multicomponent response. The demonstration concentrated on showing that the two components of the inhibitory response observed in the medial cells of the pleural ganglion are both the result of a monosynaptic connexion with the identifiable, presumably cholinergic, presynaptic neurone I.

Gradual manipulation of transmitter release from this neurone was shown to cause a progressive and correlated change in both components of this inhibitory response. This occurs whether the release is altered by calcium deprivation (see Katz, 1969) or by prolonging the presynaptic spike by intracellular injection of TEA (see Katz & Miledi, 1967; Kusano *et al.* 1967).

It must be remembered that the calcium deprivation affects transmitter release not only from presynaptic neurone I, but from all cells of the

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Legend to Fig. 11.

A and *B*, effects of hexamethonium on response of the visceral neurone R 15 to repetitive stimulation of L 10. *A*, the response during activation of the presynaptic neurone is predominantly excitatory, and a single e.p.s.p. follows each presynaptic spike. *B*, hexamethonium (10^{-3} M) has eliminated the e.p.s.p., and revealed a slowly developing biphasic response to L 10 activation. The duration of presynaptic firing is indicated by a trace over each presynaptic record. Calibration: 2 sec, 5 mV. A', B': effects of hexamethonium on response to iontophoretic injection of ACh on R 15. Note that the only detectable response to an iontophoretic injection of ACh is excitatory (A'), and is completely abolished by hexamethonium (B'). Calibration: 8 sec, 5 mV. A''–D''. A'' and B'' are recordings similar to those presented in *A* and *B*, but which are taken at a slower speed and only at -80 mV and -100 mV. The slow biphasic response that persists in the presence of hexamethonium (B''), also resists curare (C'') and curare + methylxylocholine (D''). Calibration: 8 sec, 5 mV.

ganglion. Thus it would affect any hypothetical interneurone involved in a polysynaptic pathway. Although it is unlikely that the highly correlated decreases in amplitude of the two response elements would occur if multisynaptic pathways are involved, the possibility cannot be excluded by such an experiment. An intracellular injection of TEA, on the other hand, causes a selective increase in transmitter release, affecting only the injected neurone. As was shown in Fig. 3, both the rapid and slow i.p.s.p.s of the post-synaptic response in the medial neurones increase progressively with an increasing intracellular TEA concentration (and a corresponding increase in duration of the action potential) in presynaptic neurone I. The changes in the two potentials are highly correlated, thus strongly suggesting that this neurone mediates directly both components of this response. In contrast, let us imagine what would be the anticipated effect of a TEA injection in presynaptic neurone I if an interneurone (or interneurones) intervened between the action potential of increasing duration and the post-synaptic response of increasing amplitude. With TEA injection in presynaptic neurone I, increased transmitter release from that neurone could theoretically increase firing of the hypothetical interneurone, thereby causing an increase in the response recorded in the medial cells. In fact, spikes would have to be added between each test to account for the consistent increases that are seen in the medial cell reponse. This would imply that the discharge of the hypothetical interneurone(s) would have to increase from a minimum of 1 to a minimum of 15–20 spikes in order to cause the changes in the i.p.s.p.s seen in Fig. 3. These added spikes would have to occur in the interval separating the action potential of the presynaptic neurone I and the post-synaptic response of the medial cells. The firing frequencies that this implies are, however, foreign to *Aplysia* neurones.

Furthermore, once it is accepted – in view of the latency and TEA data – that at least the rapid i.p.s.p. is mediated monosynaptically and by presynaptic neurone I (which can consequently be considered cholinergic), it must be admitted that any disynaptic effects mediated by that neurone would require the intervention of excitatory cholinergic synapses. However, all such synapses thus far studied in *Aplysia* can be blocked by hexamethonium or curare. The fact that the slow i.p.s.p. persists in the presence of either of these drugs thus strongly suggests that it could not depend upon an intervening cholinergic excitation.

A single cholinergic neurone can mediate different types of responses on different post-synaptic cells. Similar reasoning can be used to reinforce the conclusion, drawn previously by Kandel *et al.* (1967), that a single cholinergic neurone can mediate different responses in different follower cells. It seems highly unlikely that the coincident and progressive increases that

are seen in the e.p.s.p.s of one group of cells (anterior pleural neurones) with presynaptic TEA injection would occur if a supplementary interneurone was involved in the production of one or both.

Thus, it appears that a single neurone, without the intervention of an additional interneurone, mediates a multicomponent response in certain post-synaptic cells and a different response in another group of cells. Although the majority of experiments permitting this conclusion were performed using the presynaptic neurone I of the pleural ganglion, similar experiments show that the same conclusion can be drawn for a variety of cholinergic responses caused by stimulation of the identifiable cholinergic neurone (L10) of the visceral ganglion (unpublished observations).

The variety of cholinergic synaptic potentials is explained by various combinations of three independent systems, each of which is characterized by a specific receptor and a specific change in permeability. The other major conclusion drawn from this paper is that the type and complexity of the cholinergic responses observed in the different post-synaptic cells is a function of which and how many of three pharmacologically distinct receptor types are found on the post-synaptic membrane. It was already shown in the second paper of this series (Kehoe, 1972*b*) that each of three cholinergic response types observed in the pleural cells (rapid e.p.s.p., rapid i.p.s.p., slow i.p.s.p.) are due to the activation of a pharmacologically distinct receptor type. In this article it was shown that the responses of the visceral ganglion cells can likewise be broken down into components dependent upon these same three cholinergic receptors. Moreover, in all cases analysed, the effect mediated, namely excitation, rapid inhibition or slow inhibition, is invariable for a given pharmacological profile. That is, a receptor blocked by hexamethonium always mediates excitation, whereas one blocked by curare, but not affected by hexamethonium, always mediates a rapid inhibition, etc. Each of the three response types is, in turn, the result of a specific, invariable, permeability change (see Kehoe, 1972*a*).

New combinations of the three receptor types have been brought to light in the analysis of the visceral neurone responses, and explain the extensive variety of post-synaptic cholinergic responses observed in that ganglion. *Of the seven possible types of responses that could exist as a result of the various combinations of three receptors, six have been identified: e.p.s.p. alone* (*see Fig.* 4); *rapid i.p.s.p. alone* (*unpublished observation*); *e.p.s.p.+rapid i.p.s.p.* (*see Fig.* 5); *e.p.s.p.+slow i.p.s.p.* (*unpublished observation*); *rapid i.p.s.p.+slow i.p.s.p.* (*see Figs.* 1, 2 *and* 8); *and e.p.s.p.+rapid i.p.s.p.+slow i.p.s.p.* (*see Fig.* 6). The only response type that has not yet been observed is a single component response resulting from the activation of the methylxylocholine-sensitive receptor mediating the slow i.p.s.p.

One point of particular interest should be emphasized concerning the results obtained from the visceral ganglion. First, what often appeared to be monophasic responses were shown, in fact, to be a net potential resulting from the simultaneous occurrence of i.p.s.p.s and e.p.s.p.s of similar time courses. The duality of these responses can only be shown with the use of pharmacological tools. For example, in cells which bear the hexamethonium-sensitive receptor (mediating the e.p.s.p.) as well as the curare-sensitive, hexamethonium-insensitive receptor (mediating the rapid i.p.s.p.) the synaptic response is often very difficult to distinguish, electro-physiologically, from that seen in cells bearing only the hexamethonium-sensitive receptor. It is only after hexamethonium has blocked the e.p.s.p. that the presence of a simultaneously occurring rapid i.p.s.p. is revealed. It can readily be imagined that the understanding of the ionic bases of that response would be rendered difficult without the use of pharmacological tools, and without the foreknowledge that such tools existed and were necessary.

Other factors which contribute to the descriptive characteristics of a synaptic potential. First, it was shown that the amplitude of a somatically recorded potential can be expected to vary as a function of the effective distance (combined effect of resistance characteristics, actual distance, etc.) separating the point of recording from the point of origin of the potential (the synapse). Thus, the synaptic response in the giant cell (often 800 μ in diameter) is often smaller for a given presynaptic activation than is that of the medial pleural cells (250–300 μ in diameter). Secondly, the inversion potential for a given response will likewise be affected by geometric factors. If one records and polarizes in the soma, and if the synaptic region is at a significant distance from the point of recording, it can be expected that there will be a marked discrepancy in the potential recorded in the soma and that existing at the point of origin of the synaptic potential (see Ginsborg, 1967). Thus, for example, in order for the synaptic region to be brought to the potassium equilibrium potential (-80 mV) it might well be necessary to polarize the soma to -100 mV or more, and in some cases it might be impossible by means of a somatic electrode, to invert a potassium-dependent potential, particularly in cells showing marked anomalous rectification (Kandel & Tauc, 1966). This discrepancy is enhanced if the cell has been damaged slightly in preparation of the ganglion, a risk which is in fact greater for the larger cells.

An additional factor, however, complicates many of the responses observed in the visceral ganglion and is probably the factor responsible for the hyperpolarizations that cannot be inverted. Certain post-synaptic cells responding to activation of L10 appear to give so-called 'non-cholinergic' responses coincident with the cholinergic potentials triggered by activation of L10. They are defined as 'non-cholinergic'

since they are not reproduced by an ionophoretic injection of ACh, and are not blocked by any one or any combination of the blocking drugs used to eliminate the three types of responses to ACh injection. In fact this response is unmasked by the presence of these drugs, and is sometimes only barely noticeable in the normal state.

This non-cholinergic response, which is for the time being considered to be of similar origin in the many different cells, takes different forms in different neurones and at different firing frequencies of the presynaptic neurone. Although it has not been analysed in detail, it seems likely that it is transmitted by electrical synapses – perhaps those shown by Waziri (1969) to exist between L 10 and certain post-synaptic cells – or indirectly via an electrical contact with L 21 (see Waziri, 1971). The possibility that this response represents a non-cholinergic chemical synapse being driven by L 10 cannot as yet be excluded; however, the fact that it persists in curare (which blocks all excitatory cholinergic effects observed until now) would suggest that the non-cholinergic chemical synapse be driven by an electrical connexion with L 10, rather than by a chemical connexion, or that L 10 liberates, itself, more than one transmitter substance.

That a presynaptic spike or series of spikes transformed by an electrical synapse could have a wide variety of manifestations (predominantly depolarizing, predominantly hyperpolarizing, biphasic) has been suggested by data gathered on another, presumably electrical, synapse in *Aplysia* (see Tauc, 1969). Thus, the variety of shapes shown by the non-cholinergic potentials may not necessarily imply that they are of different origins for the different cells. Experiments evaluating the possible ion movements giving birth to these non-cholinergic potentials are discussed in the first paper of this series (Kehoe, 1972*a*).

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