The Ionic Permeability Changes during Acetylcholine-Induced Responses of *Aplysia* Ganglion Cells

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ABSTRACT ACh-induced depolarization (D response) in D cells markedly decreases as the external Na^+ is reduced. However, when Na^+ is completely replaced with Mg^{++}, the D response remains unchanged. When Na^+ is replaced with Tris(hydroxymethyl)aminomethane, the D response completely disappears, except for a slight decrease in membrane resistance. ACh-induced hyperpolarization (H response) in H cells is markedly depressed as the external Cl^- is reduced. Frequently, the reversal of the H response; i.e., depolarization, is observed during perfusion with Cl^--free media. In cells which show both D and H responses superimposed, it was possible to separate these responses from each other by perfusing the cells with either Na^+-free or Cl^--free Ringer's solution. High $[K^+]_0$ often caused a marked hyperpolarization in either D or H cells. This is due to the primary effect of high $[K^+]_0$ on the presynaptic inhibitory fibers. The removal of this inhibitory afferent interference by applying Nembutal readily disclosed the predicted K^+ depolarization. In perfusates containing normal $[Na^+]_0$, the effects of Ca^{++} and Mg^{++} on the activities of postsynaptic membrane were minimal, supporting the current theory that the effects of these ions on the synaptic transmission are mainly presynaptic. The possible mechanism of the hyperpolarization produced by simultaneous perfusion with both high $[K^+]_0$ and ACh in certain H cells is explained quantitatively under the assumption that ACh induces exclusively an increase in Cl^- permeability of the H membrane.

INTRODUCTION

The ionic permeability changes during synaptic excitation at the neuromuscular junction of the frog were first considered by Fatt and Katz (20) and del Castillo and Katz (14). They inferred from the conductance changes which they observed that the excitatory transmitter may cause the subsynaptic membrane to become nonspecifically permeable not only to Na^+ but also to K^+

and/or Cl^-. In mammals, Coombs, Eccles, and Fatt (11) observed the effects of intracellularly injected ions on the EPSP's of spinal motoneurons, which they interpreted as supporting evidence for this short-circuit hypothesis. This concept has also been supported by many other investigators since the reversal potentials for the excitatory postsynaptic potential obtained from varying neurons of different species were found to be approximately −20–0 mv (7, 27, 29, 30, 41, 44). In addition, Nastuk (43) and Furukawa and Furukawa (22) found in the neuromuscular junction that large molecules such as various methylammonium ions can penetrate the postsynaptic membrane when activated by transmitter. In the study of end plate current, however, Takeuchi and Takeuchi (50, 51) concluded that the Cl^- permeability did not appreciably increase during the excitatory action of the transmitter.

Since Coombs, Eccles, and Fatt (9, 10) originally analyzed the ionic permeability changes during inhibitory synaptic transmission in cat motoneurons, a number of similar papers have been published on various inhibitory synapses of mammalian nerve cells (1, 16, 17, 36). According to them, the inhibitory action of the synaptic transmitter in mammals is produced normally by a specific increase in the permeability of the postsynaptic membrane to K^+ and Cl^-. On the other hand, vagal inhibition observed in mammalian cardiac muscle has been described as due to an increase in the K^+ permeability of the muscle fiber in response to acetylcholine (6, 31, 54, 55).

The inhibitory postsynaptic potentials obtained from crustacean stretch receptor cells have been ascribed to an increase in permeability of the subsynaptic membrane to both K^+ and Cl^-, but the K^+ permeability increase is thought to play the major role (18, 19, 28, 40). On the other hand, Boistel and Fatt (4) have demonstrated that inhibition in crustacean muscle is produced almost entirely by an increase in Cl^- permeability. Similarly, Kerkut and Thomas (39) have reported that inhibition in ganglion cells of *Helix* results mainly from an increase in Cl^- permeability and to a much lesser degree from an increase in K^+ permeability. Usherwood and Grundfest (56) also confirmed the predominance of the Cl^- permeability change in the inhibitory process of the grasshopper neuromuscular junction. The ionic permeability changes during synaptic excitation or inhibition thus differ according to the species, transmitters, and receptors. One of the advantages of using *Aplysia* ganglion cells for this type of investigation is the fact that a transmitter, acetylcholine, can produce either an excitatory or an inhibitory process depending upon the nature of the receptors in the postsynaptic membrane. In the present paper, a general survey of the ionic permeability changes during cholinergic depolarization and hyperpolarization of the postsynaptic membrane of *Aplysia* ganglion cells, was made to provide basic information for further analysis of the effects of various pharmacological agents on synaptic transmission.

METHODS

Procedure

The abdominal ganglion and a pair of pleurovisceral connecting nerves of *Aplysia californica* were dissected and placed in a perfusing chamber with an appropriate fixing device. The temperature of the perfusing solution was continuously monitored by a fine thermocouple placed in the vicinity of the ganglion, and was kept approximately constant at 10° C throughout all experiments. A pair of platinum wires was placed on the pleurovisceral connecting nerves as stimulating electrodes. The effective perfusing volume of the chamber was 0.2 cc and the rate of perfusion was approximately 5 cc per min. The connective tissue covering the dorsal surface of the ganglion was carefully removed to expose the underlying ganglion cells directly to the external

TABLE I

IONIC CONCENTRATIONS OF ARTIFICIAL *Aplysia* RINGER'S AND SOMATOPLASM

Intracellular concentrations of Na^+, K^+, and Ca^{++} were obtained by atomic energy absorption spectrophotometry. Values are means of 400 cells including both D and H type cells. Cl^- concentration was measured separately in D and H type cells by means of the Ag microelectrode technique (37). Values are the means of 12 D and 11 H type cells (from unpublished data by J. Maruhashi and M. Stanier).

	Na^+	K^+	Cl^-	Ca^{++}	Mg^{++}
Artificial *Aplysia* Ringer's, mM	587	11.6	671	14	52
Somatoplasm, mM	67	232	12 (D) 43 (H)	6	—

perfusing solution. Two microelectrodes filled with 2 M potassium citrate were inserted within a single cell under binocular microscopic control. A constant current pulse (I) of 500 msec was fed through one of the electrodes at a frequency of 0.2 cps and the membrane resistance (R) was continuously recorded from another microelectrode as the potential drop (IR) across the membrane. This pulse duration was usually long enough to attain the plateau of IR since the time constant of the cell membrane ranged from 100 to 200 msec (21). The recording microelectrode was connected to a conventional cathode follower preamplifier. Membrane potentials were recorded with a Grass Polygraph (inkwriter). The time course of the change in potential or resistance produced by drugs was slow enough to insure faithful recording. A cathode ray oscilloscope (Tektronix type 502) was also used for observing the general configuration of the intracellular spike potentials.

Solutions

Cells were normally perfused with artificial *Aplysia* Ringer's for which the ionic composition is shown in Table I (3). The pH was adjusted to be 7.3–7.4 normally with

Tris(hydroxymethyl)aminomethane and HCl, and with acetic acid for Cl^--free Ringer's.

Na^+-free *Aplysia* Ringer's was usually made by replacing NaCl with equimolar Tris(hydroxymethyl)aminomethane-chloride, or occasionally with $MgCl_2$.

Cl^--free *Aplysia* Ringer's was made by replacing NaCl with equimolar Na propionate unless otherwise specifically described; KCl, $CaCl_2$, and $MgCl_2$ were replaced

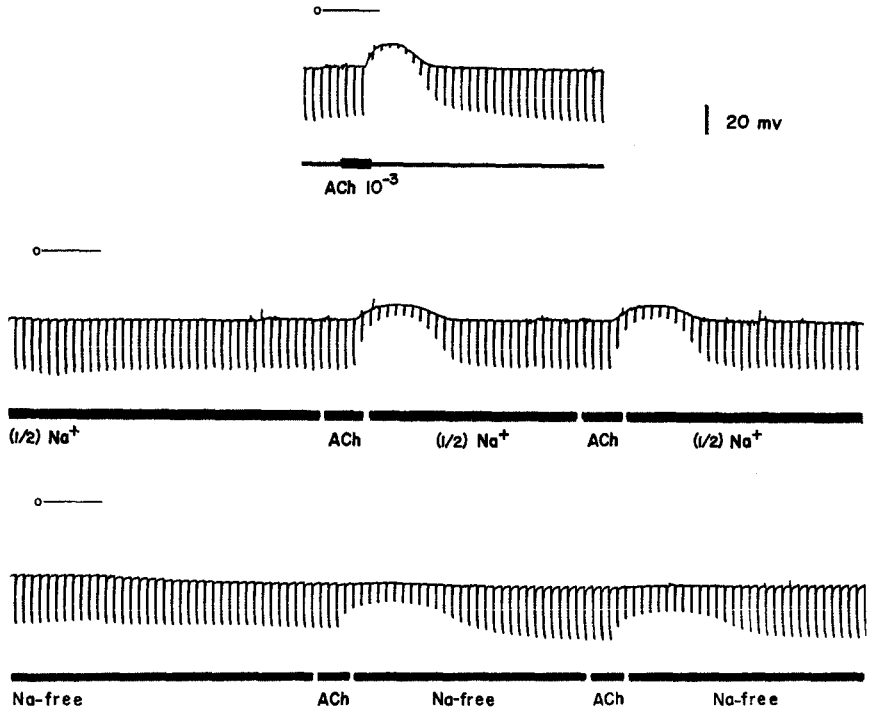

FIGURE 1. D type responses in one-half normal [Na^+] and Na^+-free Ringer's solution. Top record is a control. Downward lines emerging from the base line are the simultaneous recording of the membrane resistance (refer to Methods). Their interval is 5 sec. The horizontal bar on the left shoulder of each trace indicates extracellular potential level. The strength of ACh is expressed in grams per cubic centimeter and is kept constant at the value used in the control being dissolved in the immediately preceding solutions. This nomenclature applies to all other figures in this paper. Perfusion with one-half [Na^+] or Na^+-free Ringer's actually started 3 min prior to each recording shown in the second and third rows. Tris was substituted for Na^+.

by equimolar $MgSO_4$ to give a solution which was both K^+- and Ca^{++}-free. The side effects of K^+- and Ca^{++}-free Ringer's may be of concern. However, they were found to be very minor as far as the ACh responses (D and H) are concerned, a point which will be discussed below (see Fig. 4 and Fig. 12 under Results). When Ca^{++} is removed from the perfusing solution, the frequency of spontaneous spike activity gradually increases and the membrane resting potential depolarizes slightly with increased background synaptic activity. However, this effect is greatly reduced when excessive Mg^{++} is

added. Other large anion salts such as acetylglycine, monosodium glutamate, and pyroglutamate were used as substitutes for Cl^-. The results were generally the same as those obtained with propionate.

The tonicity of each solution was measured by an osmometer and adjusted to be isotonic with the normal *Aplysia* Ringer's by adding sucrose or urea. High $[K^+]$ *Aplysia* Ringer's was usually made by mixing the isotonic KCl Ringer with normal Ringer's for a desired level of $[K^+]$. Thus the tonicity was kept constant.

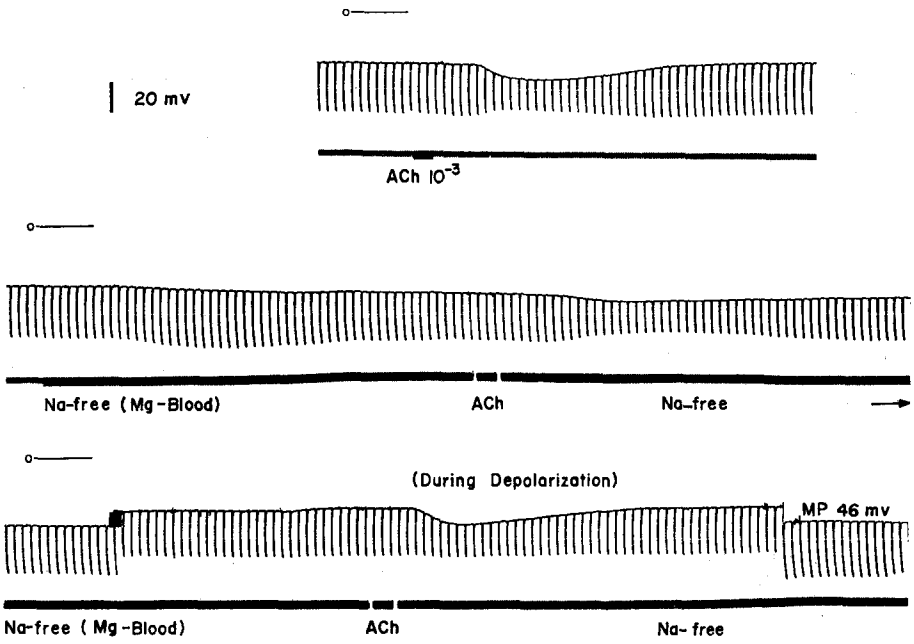

FIGURE 2. H type responses in Na^+-free Ringer's solution. Na^+ was replaced by Mg^{++}. Third row continues immediately after the second row. The membrane potential (MP) was hyperpolarized as much as 10 mv during the perfusion with Na^+-free Ringer's. Bottom recording was made during the artificial depolarization to compensate for the above hyperpolarization.

Determination of Membrane Type (D and H)

Iontophoretic application of ACh to the exposed cell surface was first used to identify the type of cell, but was later replaced by the simpler and quantitatively more accurate external perfusing technique, since the result was practically the same if dilute ACh of 10^{-6} g/cc was added to the external test solution. The former technique had some advantage in isolating the effect of ACh on a single cell, but it also had certain practical disadvantages. Primarily this was because of the difficulty in evaluating the actual concentration of ACh on the soma surface for a given quantity of applied current, since the cells were continuously perfused throughout the experiment. In addition, the amplitude and time course of the ACh-induced response with a given quantity of current often did not remain constant for the time required for the

observation. This is probably due either to a slight movement of the cell during the exchange of perfusing media from one to another, or to the period of waiting until new ionic equilibrium was reached.

There are two features which are quite helpful in determining the type of cells before applying ACh. One is their location and the other is their color. Dark brown cells are localized in the center of the right half of the dorsal surface and often found to be typical D cells, whereas light yellow cells are distributed in the periphery of the left half of the dorsal surface and are mainly typical H cells. There is a group of cells

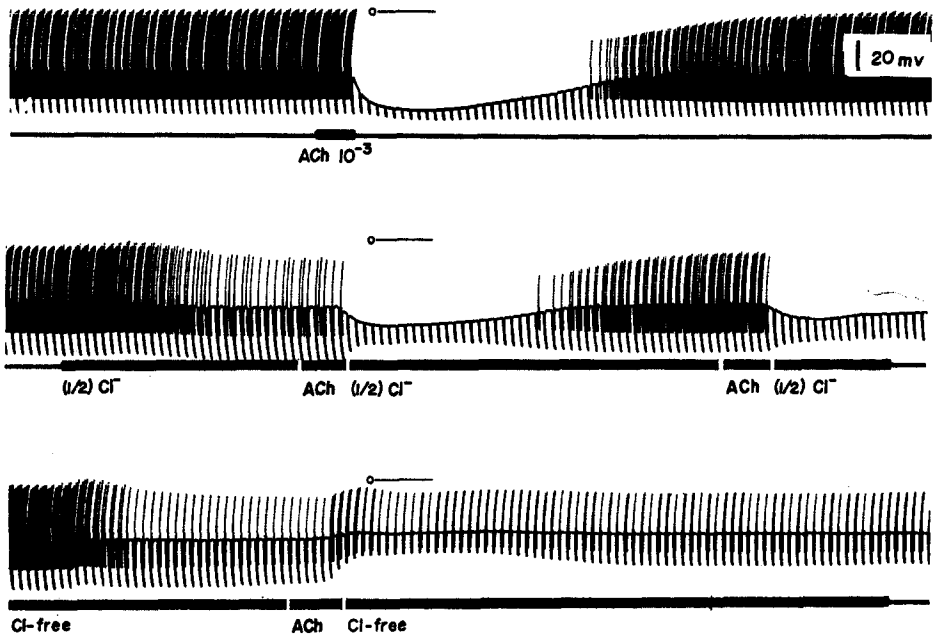

FIGURE 3. H type responses in reduced external [Cl^-] and Cl^--free Ringer's solution. Cl^--free perfusion actually started 1 min prior to the recording shown at the bottom. Note that this Cl^--free solution is K^+-free at the same time. Propionate was substituted for Cl^-.

located in the left caudal half of the dorsal surface, which appear to be yellow but are dotted with a few brown spots. The ACh responses of these cells are generally characterized as follows: they usually respond to dilute ACh (10^{-6} g/cc) as a pure H type membrane, showing monophasic hyperpolarization, but they respond to concentrated ACh (10^{-3} g/cc) as a mixed response of H and D type membranes, showing diphasic or triphasic change in the membrane potential. Using the iontophoretic application of ACh, Wachtel and Kandel (57) recently found a certain cell (L7) in the abdominal ganglion of *Aplysia*, which has both cholinergic excitatory and inhibitory receptor properties in the same cell. For convenience, we have called these cells "H-D type" although the secondary effect from the neighboring cholinergic neurons could not be excluded.

From pharmacological observations, Gerschenfeld and Tauc (25) have extended the original classification of D and H type gastropod ganglion cells. They now include D, DILDA, and DINHI for D type cells; and H and HILDA for H type cells. Since we did not further subdivide the pharmacological nature of our D or H type cells, the results presented here should not be generalized for all types of cells. However, the cells described as D type in this paper are mainly the DILDA type of Gerschenfeld and Tauc since these cells had both spontaneous EPSP's and IPSP's and their IPSP's were not affected by the external $[K^+]$. The pure D type cells of their classification are very rare in the abdominal ganglion of *Aplysia*. Our H type cells are either their H or HILDA type cells since some of our H type cells had spontaneous IPSP's with long duration. More important, however, is the fact that the results obtained on our H type cells were consistent, and were independent of the occurrence of IPSP's of long duration.

RESULTS

Na^+ in Cholinergic D and H Responses

The ACh-induced D response is markedly influenced by external Na^+ concentration. As shown in Fig. 1, the amplitude gradually decreases as the external Na^+ is reduced. This is usually associated with gradual membrane hyperpolarization. Accordingly, the actual decrease in amplitude of the D response is apparently reduced by this parallel change. Another significant point is the change in membrane resistance. The membrane resistance during the control D response is approximately $\frac{1}{20}$ of the resting resistance. When external Na^+ is reduced to $\frac{1}{2}$ of the normal concentration, the decrease in resistance during the D response is reduced to approximately $\frac{1}{5}$ of the resting resistance. In Na^+-free solution, the resistance is reduced to only $\frac{1}{2}$ of the resting resistance.

These findings suggest that the ACh-induced depolarization in normal *Aplysia* Ringer's is produced mainly by an increase in Na^+ permeability of the subsynaptic membrane. However, it is worthy of note that the membrane resistance still decreases to $\frac{1}{2}$ of the resting value in response to ACh, when practically no Na^+ is in the external media. This will be discussed later.

The ACh-induced H response is not appreciably altered by external Na^+ concentration, as is shown in Fig. 2. The amplitude of the H response seems to be reduced, apparently because of gradual membrane hyperpolarization caused by Na^+-free media. However, one can easily visualize that the H response is not significantly affected during Na^+-free perfusion when the hyperpolarization due to Na^+-free media is compensated for by an artificial depolarization (see the bottom trace in Fig. 2). In this experiment, Na^+ was replaced by Mg^{++} which indicates that the H response is not influenced by excessive Mg^{++} in the external solution.

Low Cl^- Effects on H and D Responses

The ACh-induced H response is greatly influenced by the external Cl^- concentration. As shown in Fig. 3, the amplitude gradually decreases as the ex

ternal Cl^- is reduced. In Cl^--free media it often reverses to depolarization. The resting membrane potential is not appreciably altered by Cl^--free perfusion, if propionate or pyroglutamate is used to replace Cl^-. This reversal of H response is therefore not due to a change in resting potential. The membrane resistance during the control H response decreased to approximately $\frac{1}{5}$ of the resting resistance. When the external Cl^- was reduced to $\frac{1}{2}$ of the normal concentration, the membrane resistance decreased to approximately $\frac{1}{3}$ of the resting resistance. In Cl^--free perfusion, the membrane resistance decreased to only $\frac{1}{2}$ of the resting resistance. This parallel change in the membrane conductance with the varying external Cl^- concentrations strongly suggests that an increase in Cl^- permeability is the major cause of ACh-induced hyperpolarization.

Reversal of the H response does not occur in all H cells, as shown in Fig. 4. In this case, the decrease in membrane resistance during the H response is greatly reduced in Cl^--free media, being less than 10% of the resting resistance. Whether the reversal of H response occurs in Cl^--free media or not may depend entirely upon the original intracellular Cl^- concentration. Absence of reversal in these cells is probably due to a very low concentration of intracellular Cl^-.

The effects of Cl^--free media on D and H cells are shown in Fig. 5. The D response appeared to be depressed a little in Cl^--free media. However, it should be noted that the membrane resting potential is often depolarized with prolonged Cl^--free perfusion. If this depolarization is taken into consideration, the depression of the D response in Cl^--free media is very slight. On the other hand, the H response clearly reversed to depolarization in Cl^--free media.

Low Na^+ or Cl^- Effects on H-D Responses

There are peculiar cells, H-D type (see Methods), which show both H and D type responses to ACh of 10^{-3} g/cc. It was concluded above that the D and H responses observed in pure D and H cells from due mainly to increased Na^+ and Cl^- influxes through each subsynaptic membrane. If this is valid, one should be able to separate the H response from the D response of the H-D cell by perfusing with Na^+-free or Cl^--free solution. The results are shown in Fig. 6. As was expected, the D response disappeared in Na^+-free media, whereas in Cl^--free media, the H response reversed to depolarization and overlapped the D response. Recently Gerschenfeld (23, 24) reported that the noncholinergic inhibitory process in the land snail ganglion is produced exclusively by an increase in the K^+ permeability of the subsynaptic membrane. If this is the case, all our H-D cells must originally belong to the cholinergic H type, since their H responses are quite sensitive to the external Cl^- concentration.

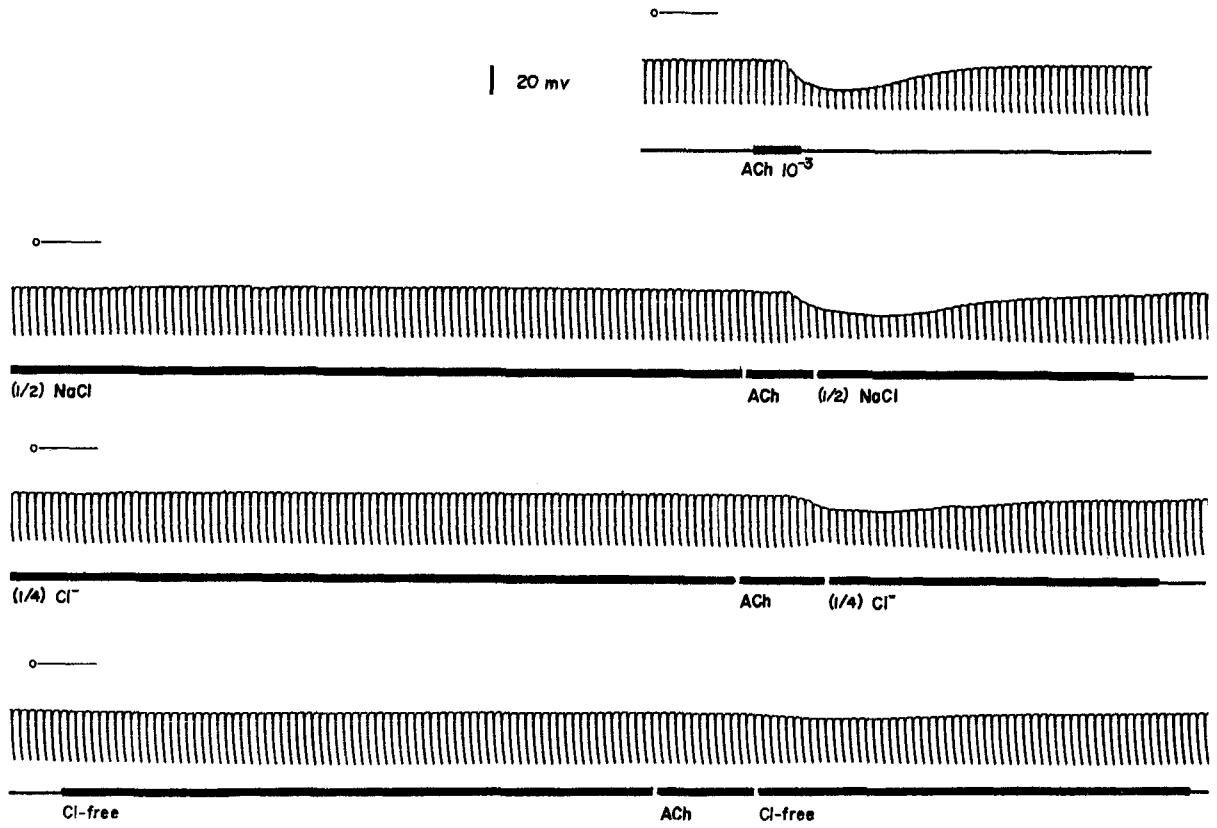

FIGURE 4. A particular H type response which does not show the reversal of the hyperpolarizing response to the depolarizing one in Cl^--free media. Note that the conductance increase in response to ACh as well as the hyperpolarization, is markedly depressed in Cl^--free media.

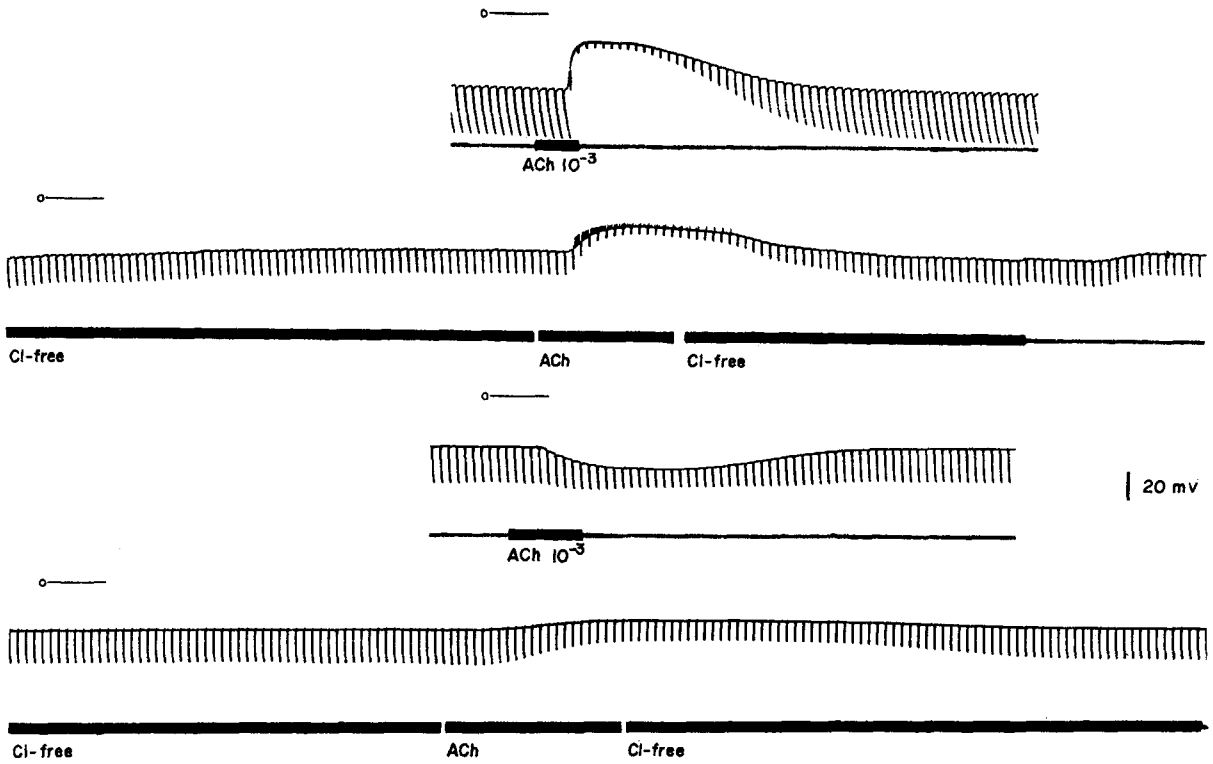

FIGURE 5. D and H type responses in Cl^--free Ringer's solution. Cl^--free perfusion actually started 1 min prior to the record shown in the second row and 2 min prior to the record shown in the bottom row. The top and the third rows are the controls obtained from D and H type cells, respectively. The apparent decrease in resting resistance in the second row is not due to Cl^--free solution but to an artifact.

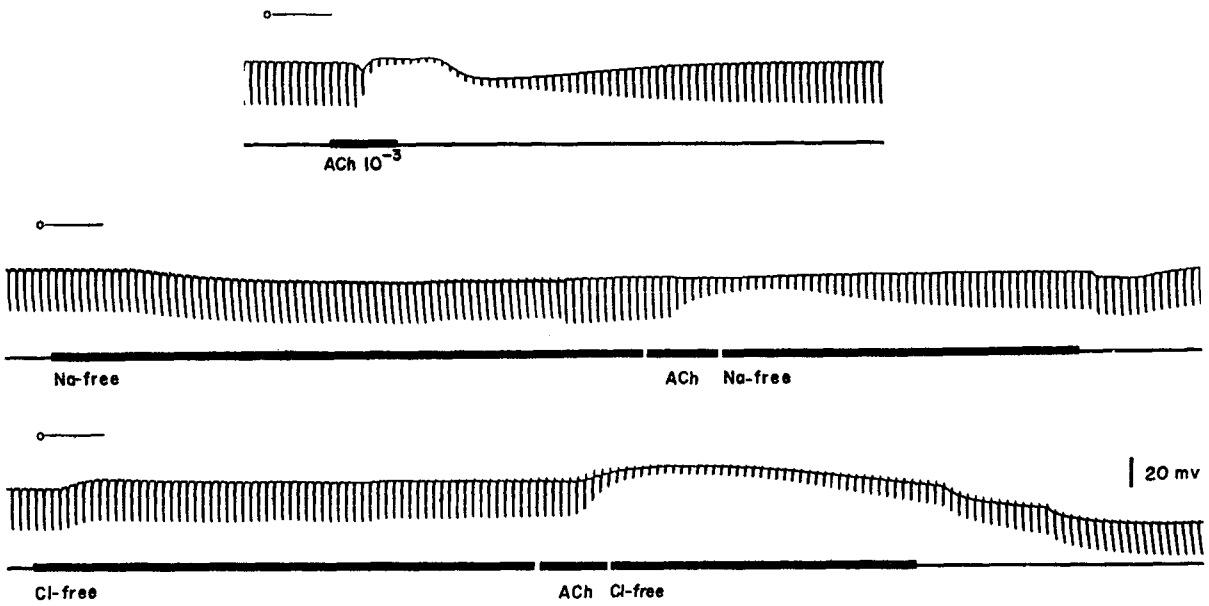

FIGURE 6. Effects of Na^+-free and Cl^--free perfusions on the mixed type response. Control response shown at the top consists of an H type response superimposed by a D type response. Note that these responses are isolated from each other by perfusing either with Na^+-free or with Cl^--free Ringer's.

Whether the D response of H-D cells is also cholinergic remains a question, however its depolarization is sensitive to the external Na^+ concentration as is the cholinergic response.

High K^+ Effects on D and H Responses

When our present method of perfusion was used, not only the cell under study but also some presynaptic nerves and other cells in the vicinity of the cell were

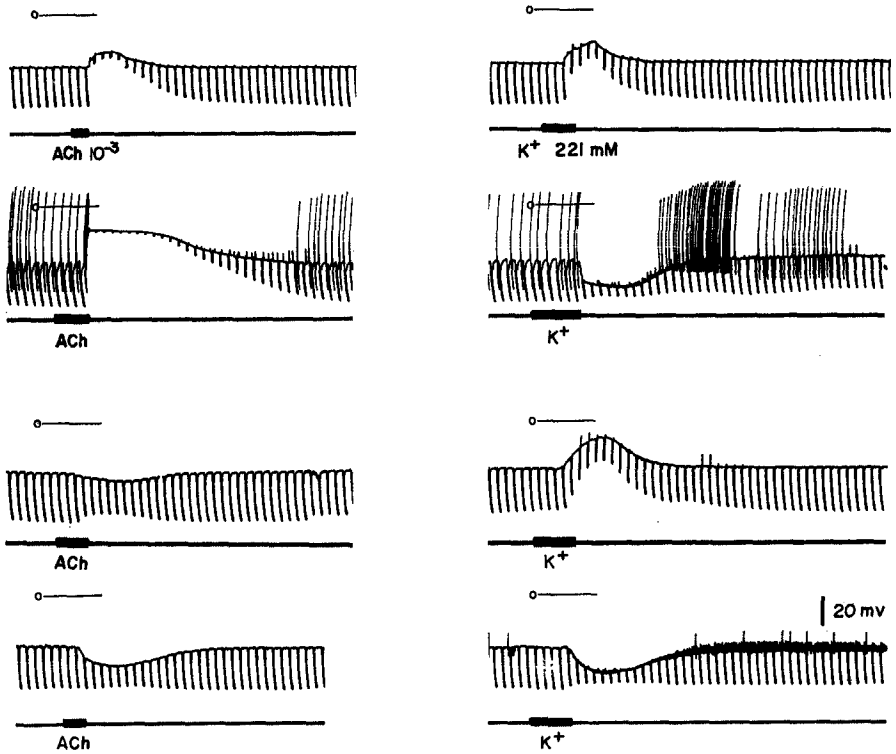

FIGURE 7. Effects of high $[K^+]_0$ on D type and H type cells. A pair of recordings in the same row were obtained from the same cell. Note that the high $[K^+]_0$ depolarizes or hyperpolarizes the cell regardless of whether it is a D or an H type. Concentrations of K^+ (mM) and ACh used in the lower traces are the same as those shown at the top.

simultaneously stimulated by the high $[K^+]_0$ solution. Accordingly, the effect of $[K^+]_0$ on a given cell is often complicated by an inhibitory or excitatory, or combined synaptic activities (see Fig. 7). The membrane potential change produced by high external $[K^+]$ often does not follow the theoretical Nernst equation. However, if the cell is treated with Nembutal, and thus isolated from the action of nerves which impinge upon it, the cell is readily depolarized much as expected from the Nernst equation, regardless of cell type. This is good evidence that the K^+ has reached the cell membrane from which the

recording was made (Fig. 8). Barbiturates are known to block synaptic transmission in the central nervous system (5, 42, 47, 48). We have confirmed in our laboratory that Nembutal of 6×10^{-3} g/cc in concentration blocks both ACh-induced D and H responses (45, 46). The important observation here is that the expected K^+ depolarization was entirely suppressed by an intense inhibitory bombardment before the use of Nembutal, and this inhibitory activity was sufficient to hyperpolarize the membrane as much as 20 mv in spite of a high external $[K^+]$ of 221 mM (Figs. 7 and 8).

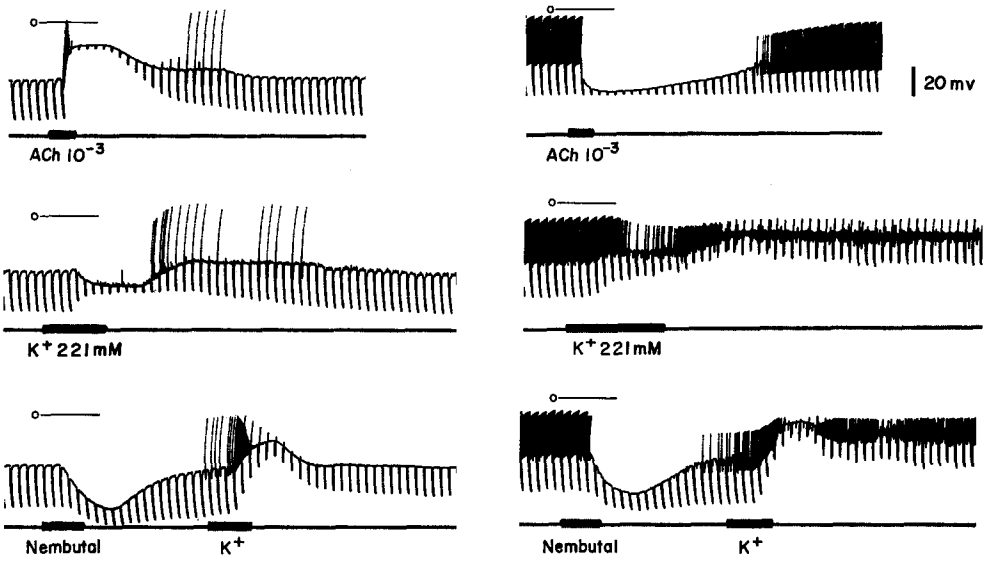

FIGURE 8. Disclosure or enhancement of the K^+ depolarization observed in a D type cell (left column) and an H type cell (right column), following application of Nembutal 6×10^{-3} g/cc. K^+ concentration in bottom traces is the same as that used in the controls shown in the middle row.

There are some D and H cells in a ganglion which receive a minimal inhibitory innervation. Such cells were readily depolarized by high external $[K^+]$ perfusion almost as predicted from the Nernst equation, without applying Nembutal. In these cells, it was possible to observe the mutual interference between ACh and high external $[K^+]$ effects on the soma membrane in relative isolation from the presynaptic disturbance (Fig. 9). In H cells, the effect of ACh and high $[K^+]_o$ depends on which is applied first. When ACh is applied first, the normal K^+ depolarization is markedly depressed. Similarly, when high $[K^+]_o$ is applied first, the normal ACh hyperpolarization is significantly suppressed. When ACh and high $[K^+]_o$ are applied at the same time, the K^+ depolarization is completely suppressed, whereas the ACh hyperpolarization is only slightly depressed. It should be noted that the resistance decrease in

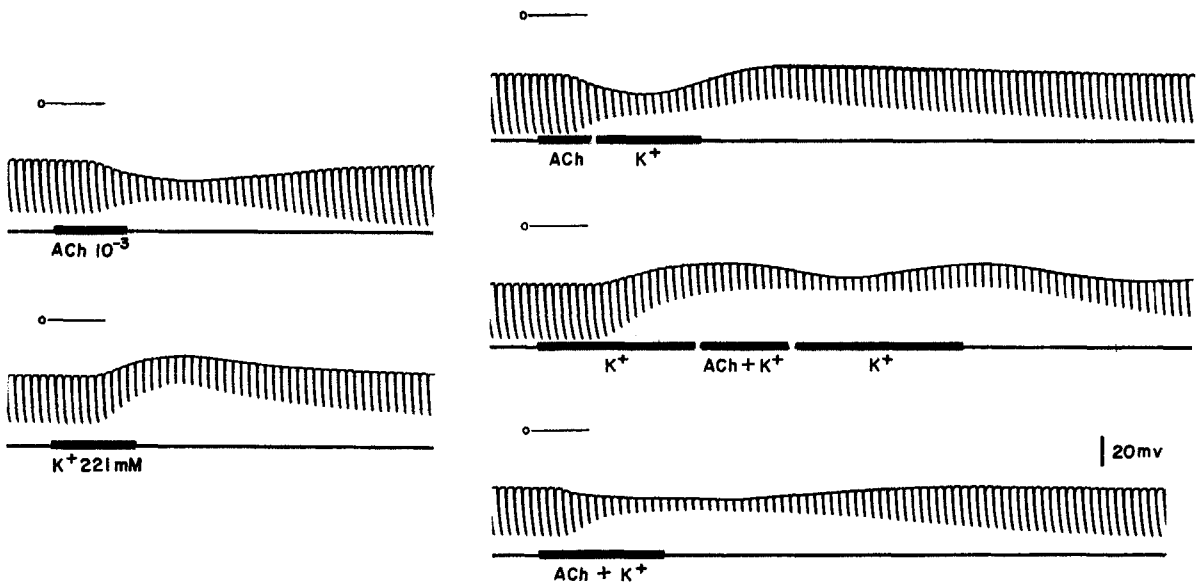

FIGURE 9. Interference between ACh-induced hyperpolarization and K^+-induced depolarization observed in an H type cell. Note that the increase in membrane conductance observed at the bottom right is greater than either one of the control responses shown at the left, in spite of smaller hyperpolarization.

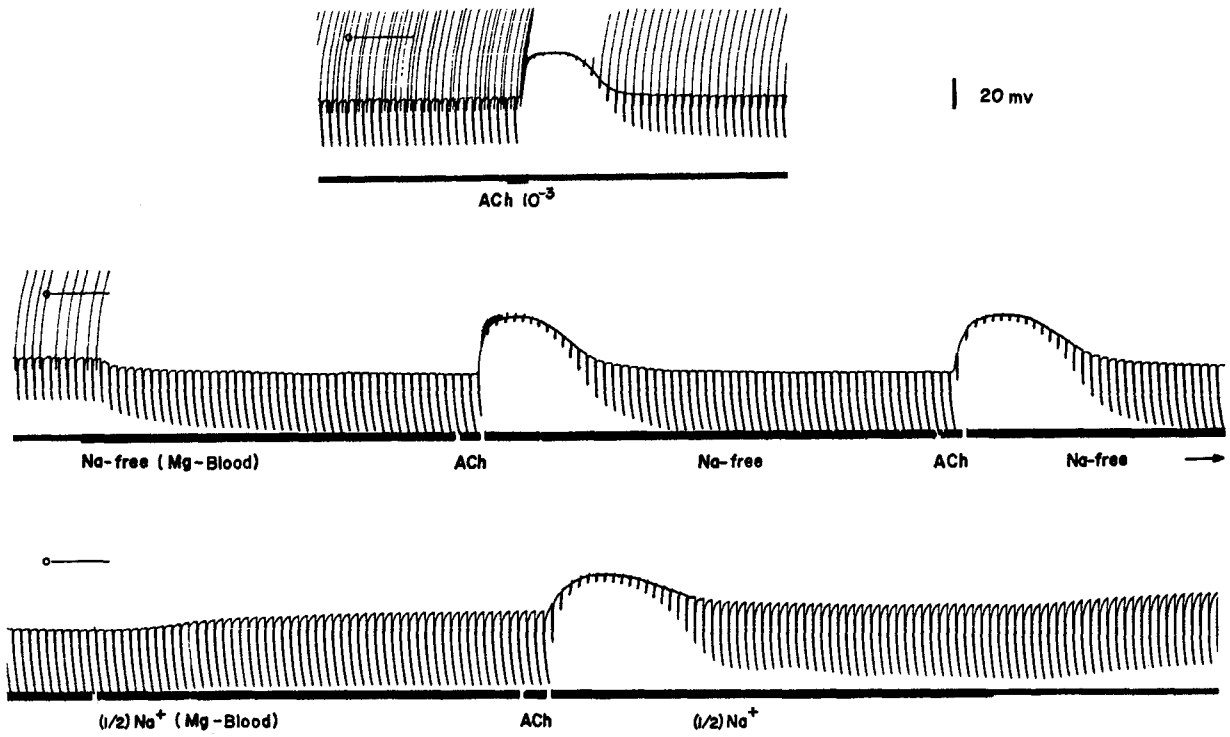

FIGURE 10. D type responses in Na^+-free and Mg^{++}-rich Ringer's solution. Total NaCl was replaced by the isotonic $MgCl_2$. Bottom trace immediately follows the middle trace. Note the membrane potential change due to Na^+-free perfusion.

response to simultaneous perfusion with both ACh and high $[K^+]_0$ was greater than that produced by either ACh or high $[K^+]_0$ alone (Fig. 9).

High and Low Mg^{++} Effects on D and H Responses

In experiments with Na^+-free perfusion, ACh depolarization was entirely abolished, but ACh still appreciably decreased the membrane resistance. This suggested that the primary action of ACh is to increase the permeability of the postsynaptic membrane not only to Na^+ but also to other ions. Normal *Aplysia* Ringer's contains Mg^{++} in a concentration of 8.9% of $[Na^+]_0$. The resistance decrease induced by ACh in Na^+-free media could be due to an increase in Mg^{++} permeability at the postsynaptic membrane. In order to test this possibility, the ACh response was examined in Mg^{++}-Ringer's which was made by replacing the total NaCl of normal Ringer's with isotonic $MgCl_2$. As shown in Fig. 10, ACh depolarization remained nearly the same as the control response despite the absence of Na^+. Gradual membrane hyperpolarization, an increase in resistance, and disappearance of spontaneous activity are consistently observed in Na^+-free perfusion, regardless of the substitutions made. These changes in membrane resting potential and resistance should again be taken into consideration when the ACh responses are compared before and after the Na^+-free perfusion. The results clearly demonstrate that the postsynaptic membrane becomes highly permeable to Mg^{++} during ACh depolarization.

The effects of Mg^{++}-free solutions on ACh responses were compared in D and H cells. As shown in Fig. 11, neither one showed appreciable change during perfusion with Mg^{++}-free solution. This indicates that the participation of Mg^{++} in the normal ACh responses is minimal when sufficient Na^+ and Cl^- are in the external media.

High and Low Ca^{++} Effects on D and H Responses

In the vertebrate neuromuscular junction, the amplitude of the end plate potential (EPP) is augmented by high external Ca^{++} (13, 15, 34). This increase has been ascribed to the increased liberation of transmitter substance from the nerve endings (32, 35). This Ca^{++} effect is inhibited by the presence of Mg^{++} and is believed to reflect "competitive inhibition" (12, 34). The effect of external Ca^{++} on the postsynaptic membrane was tested on both D and H cells of *Aplysia*. As shown in Fig. 12, neither the D nor the H response to ACh showed any significant change in either Ca^{++}-free or Ca^{++}-rich (four or five times normal) media. Prolonged perfusion with Ca^{++}-free or Ca^{++}-rich solution caused those changes which would be expected from the known effects of Ca^{++} on the neuronal membrane (see Fig. 12), namely, gradual fall in resting potential with decrease in resistance and spike height with lowered firing threshold in Ca^{++}-free perfusion. In Ca^{++}-rich media gradual hyperpolarization, increase in resistance and spike height, and elevated firing thresh-

old were seen (2). Nevertheless, the ACh response was not significantly altered by perfusion with either Ca^{++}-free or Ca^{++}-rich media. Thus the activity of the postsynaptic membrane of *Aplysia* is not significantly influenced by the external Ca^{++} concentration, provided that sufficient Na^{+} is in the external media. Takeuchi (49) reported that the ACh-induced end plate current, recorded from the frog's neuromuscular junction with voltage clamp technique

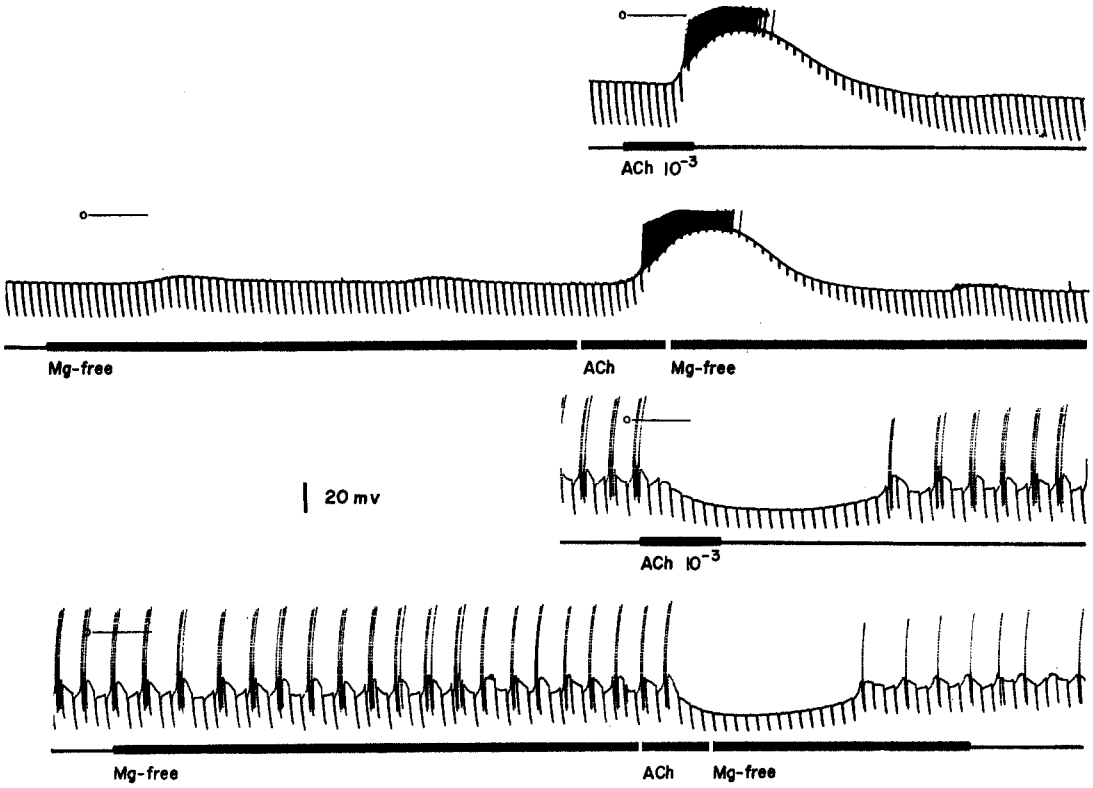

FIGURE 11. D and H type responses in Mg^{++}-free Ringer's solution. Top and third recordings are controls obtained from D and H type cells, respectively.

was depressed by 28% in 15 times normal Ca^{++} solution. Therefore, any augmentation of EPP or EPSP observed when $[Ca^{++}]_0$ is increased may be due entirely to the change in the presynaptic level including the process of ACh liberation.

Were Ca^{++} to inactivate choline esterase, the ACh response would be expected to be augmented or prolonged in Ca^{++}-rich media. This possibility appears to have been excluded. However, Ca^{++} within a postsynaptic membrane of the marine animals may be fixed so stably that the change in $[Ca^{++}]_0$ does not alter the effective $[Ca^{++}]_0$ unless a chelating agent is used.

DISCUSSION

Ionic Permeability Change during ACh-Induced Depolarization

In the present experiment, the membrane depolarization and the resistance decrease in response to ACh were significantly decreased when the $[Na^+]_o$

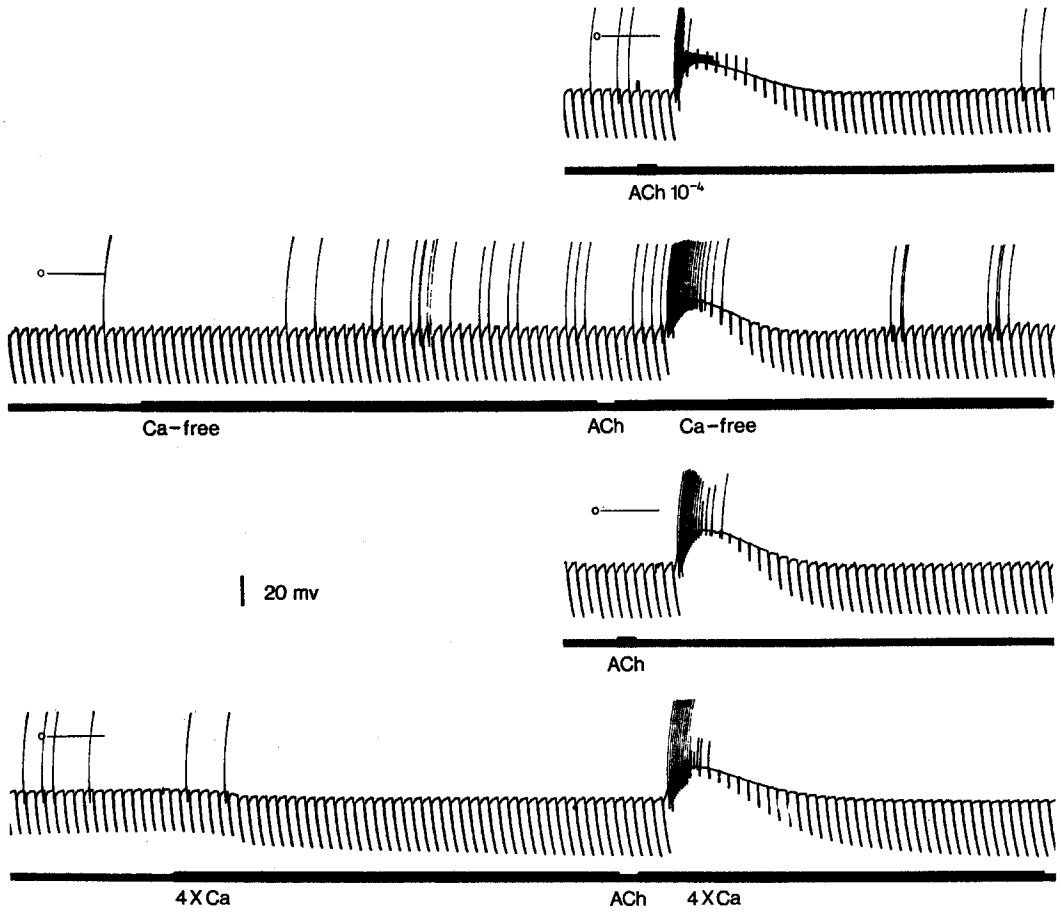

FIGURE 12 A. D type responses in Ca^{++}-free and Ca^{++}-rich Ringer's solution. 4 × Ca^{++} denotes four times normal Ca^{++} concentration; i.e., 4 × 13.87 mM. Third trace is another control taken 5 min after the recording shown in the second row.

was reduced. This parallelism strongly suggests that in normal *Aplysia* Ringer's Na^+ plays a major role during the ACh depolarization. In a Na^+-free media, it is expected that the ACh depolarization would reverse into hyperpolarization, since intracellular Na^+ should diffuse out along the concentration gradient. However, the membrane is often greatly hyperpolarized in Na^+-free solution by as much as 10–20 mv, and the membrane resistance usually in-

creases up to 1.5 times normal value. The conductance increase during the ACh response is greatly reduced during prolonged perfusion with Na^+-free solution. In a few cells, however, a significant decrease in membrane resistance in response to ACh was observed in Na^+-free media, as shown in Fig. 1. In these cases, the membrane potential remained unchanged at the originally

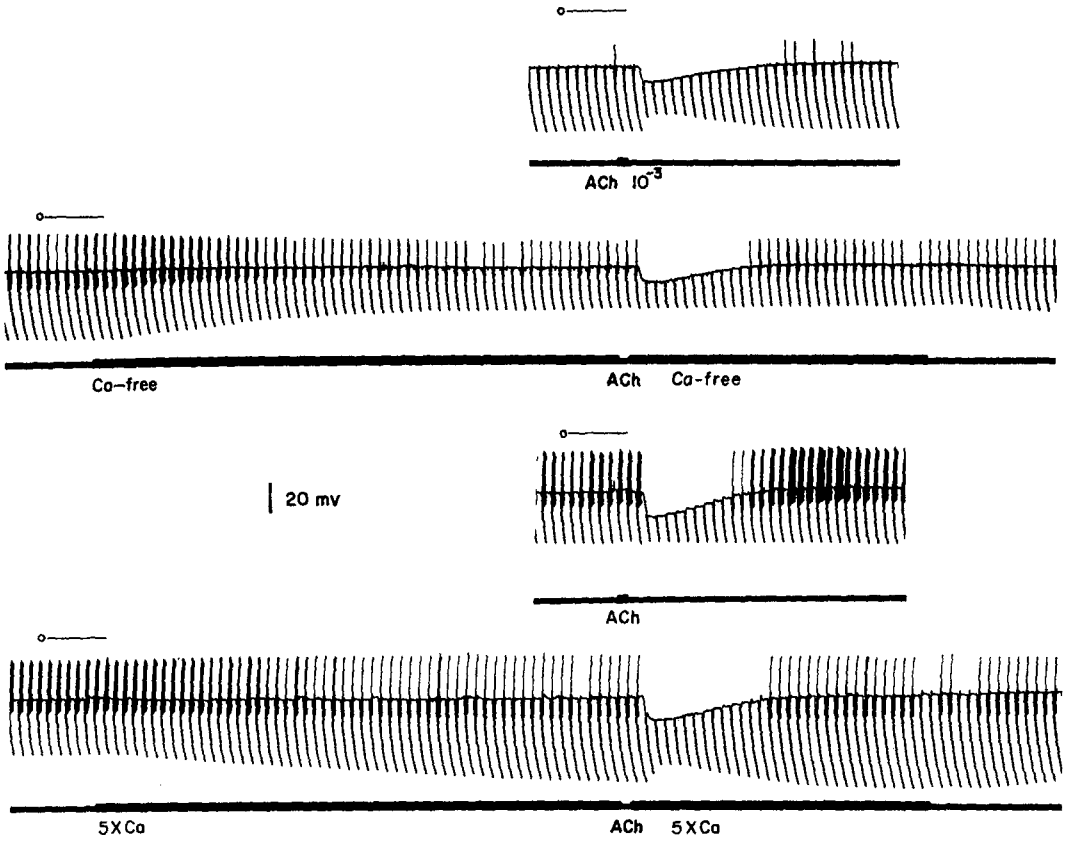

FIGURE 12 B. H type responses in Ca^{++}-free and Ca^{++}-rich Ringer's solution. Note the gradual change in membrane potential and membrane resistance in response to Ca^{++}-free and Ca^{++}-rich Ringer's perfusion.

hyperpolarized level. If the membrane were not hyperpolarized, one would have seen the reversal in response to ACh in a Na^+-free media. In addition, Takeuchi (49) reported that ACh produces a considerable increase in Ca^{++} permeability when $[Na^+]_o$ is greatly reduced. In our experiments, Mg^{++} was found to be a good substitute for Na^+. Therefore, in Na^+-free media, the reversal of ACh depolarization would be further impeded by these divalent cations moving into the cell.

ACh depolarization was slightly depressed in Cl^--free media (Fig. 5). (Note that our Cl^--free media are also K^+-free). If the ACh made the D type mem-

brane permeable to Cl^- as well as to Na^+, the ACh depolarization should be greatly enhanced in Cl^--free media. It is, therefore, very unlikely that any increase in Cl^- permeability contributes to the normal ACh depolarization. Takeuchi and Takeuchi (50, 51) have also concluded that the Cl^- permeability increase is minimal in amphibian end plate potentials. Recently, Kerkut and Meech (38) have also reported that the ACh-induced depolarization of snail ganglion cells is not affected by the external Cl^- concentration. A slight decrease in ACh depolarization in our Cl^--free media may be due to a K^+ permeability increase since the equilibrium potential for K^+ was increased in K^+–Cl^--free media.

Ionic Permeability Change during ACh-Induced Hyperpolarization

The inhibitory process in the ganglion cells of *Aplysia* has been studied by Tauc (52), Tauc and Gerschenfeld (53), and Chiarandini and Gerschenfeld (8). From their observations on the reversal potential of IPSP, they suggested that the IPSP produced by a cholinergic transmitter is due to the permeability increase of the subsynaptic membrane to both K^+ and Cl^-, whereas the IPSP produced by a noncholinergic transmitter is due to an increase in permeability exclusively to K^+ (24). In the present experiment, ACh hyperpolarization decreased almost linearly with decrease in $[Cl^-]_o$ until it reversed into depolarization in Cl^--free media. If the H type membrane became highly permeable to K^+ as well as Cl^- during the ACh hyperpolarization, the H response would not reverse in Cl^--free media since our Cl^--free solution is always K^+-free at the same time. In addition, it should be noted that in a few H cells which did not show the reversal in Cl^--free media, the conductance increase during the H response was almost completely abolished in K^+–Cl^--free media (Fig. 4). These results strongly suggest that the cholinergic inhibition in the abdominal ganglion cells of *Aplysia* is due mainly to the increase in Cl^- permeability and that there is very little if any increase in K^+ permeability.

In addition, one should note that the cell shown at the left of Fig. 8 is a typical D type cell. Therefore, the inhibitory synaptic transmitter causing the marked hyperpolarization during high $[K^+]_o$ perfusion must be of a noncholinergic nature. If this noncholinergic inhibition is exclusively due to an increase in K^+ permeability of the postsynaptic membrane, as suggested by Gerschenfeld and Chiarandini (24), for DINHI type cells, it should be reversed into depolarization or at least should disappear when perfused with high external $[K^+]$ of 220 mM. The marked hyperpolarization of nearly 15 mv which was observed suggests that the ionic mechanism underlying the inhibition in our D type cells differs from that in the DINHI type cells described by Gerschenfeld and Chiarandini.

Interference between K^+ Depolarization and ACh Hyperpolarization (H Cells)

One of the characteristic observations made in this paper is the fact that the K^+ depolarization of H cells due to high $[K^+]_0$ (= 220 mM), is completely suppressed during ACh hyperpolarization. We have attempted to interpret this observation from the classical concept of membrane permeability (26, 33) by introducing our findings that ACh selectively increases Cl^- permeability (P_{Cl}) but does not affect other ionic permeabilities (P_K, P_{Na}) of the H type membrane. If ACh of a given concentration increases P_{Cl} by a factor of X times the normal value, but does not alter P_K or P_{Na}, X can be expressed by the observed resistance change (R_0/R) as shown below.

TABLE II

CHANGES IN MEMBRANE RESTING POTENTIAL OF AN H TYPE CELL, PRODUCED BY VARYING CONCENTRATION OF ACh AND EXTERNAL K^+

The relative resistance changes (R/R_o) in response to ACh of 10^{-5} and 10^{-3} g/cc were ⅔ and ¼, respectively.

Resting potential E_o = −36 mv at temperature 10°C	E − E_o	
	Calculated	Recorded
	mv	*mv*
1. ACh 10^{-5} g/cc	−12	−10
2. ACh 10^{-3} g/cc	−23	−21
3. $[K^+]_0$ 120 mM	+15	+13
4. $[K^+]_0$ 220 mM	+25	+22
1 and 3 combined	−2	−2
1 and 4 combined	+8	+6
2 and 3 combined	−17	−16
2 and 4 combined	−11	−10

$$X = \frac{P_K + P_{Na}}{P_{Cl}}\left(\frac{R_o}{R} - 1\right) + \frac{R_o}{R}$$

where P_K, P_{Na}, and P_{Cl} are the permeabilities of the resting membrane to K^+, Na^+, and Cl^-, respectively. The change in membrane potential ($\Delta E = E - E_0$) in response to the varying concentrations of ACh and high $[K^+]_0$ can be calculated from the Goldman (26) and Hodgkin and Katz equation (33) by inserting the above relation and the concentrations of K^+, Na^+, and Cl^-. Normal intra- and extracellular concentrations of K^+, Na^+, and Cl^- were actually measured and are shown in Table I. For the approximation of ΔE, the ratios $P_K : P_{Na} : P_{Cl}$ of the resting membrane were assumed to be 1:0.15:0.45 in the case of the normal Ringer perfusion. This assumption was based on our unpublished observations that P_{Na} of *Aplysia* ganglion cells is approxi-

mately three times greater than that of squid giant axon (33), whereas P_K and P_{Cl} are approximately the same as those of the squid giant axon. In the case of high $[K^+]_o$ perfusion, the ratios were corrected to be 1:0.08:0.3 because of an increase in P_K due to the depolarization (33). The values of ΔE, thus calculated from the relative conductance changes for varying [ACh] and high $[K^+]_o$, were compared with those actually obtained in the potential recordings, and are shown in Table II.

In spite of the greatly simplified assumptions, the calculated values of ΔE are reasonably consistent with those obtained experimentally. This correlation lends further support to our conclusion that the cholinergic inhibitory process in the abdominal ganglion cells of *Aplysia* is exclusively due to the Cl^- permeability change.

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