J. Physiol. (1974), **242**, *pp.* 453–470
With 9 text-figures
Printed in Great Britain

INTRACELLULAR CHLORIDE ACTIVITY AND THE EFFECTS OF ACETYLCHOLINE IN SNAIL NEURONES

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(*Received 4 April 1974*)

SUMMARY

1. Cl^--sensitive micro-electrodes were used to measure intracellular Cl^- in snail neurones. The electrodes consisted of a sharpened and chlorided silver wire mounted inside a glass micropipette.

2. The electrodes appeared to record changes in internal Cl^- accurately but in H cells the chloride equilibrium potential (E_{Cl}) as measured by the Cl^--sensitive electrode was always less negative than E_{ACh}.

3. In some H cells ACh caused a measurable increase in internal Cl^- when the cell was at its resting potential. In voltage-clamped cells there was a close correlation between the change in internal Cl^- and the extra clamp current caused by a brief application of ACh. This confirmed that ACh increases the cell's membrane permeability only to Cl^- ions, and that E_{ACh} was equal to E_{Cl}.

4. There was good agreement between the measured change in internal Cl^- and that calculated from the cell size and clamp charge only when it was assumed that a constant voltage offset was added to the potential of the Cl^--sensitive electrode while it was inside the nerve cell.

5. Cl^--sensitive electrodes with AgCl as the sensitive material appear to be unsuitable for intracellular measurement of Cl^-, although they might be suitable for following changes in E_{Cl}.

6. In certain D cells ACh also caused an increase in internal Cl^- although it decreased the membrane potential. In the presence of hexamethonium, ACh caused a hyperpolarization and a smaller increase in internal chloride.

7. It is concluded that the intracellular Cl^- in both H and D cells is about 8·3 mM, giving an E_{Cl} of about -58 mV.

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INTRODUCTION

It has been known for some time that at many inhibitory synapses the transmitter substance increases the Cl^- permeability and that this, in molluscan neurones at least, leads to a hyperpolarization of the cell. This implies that the Cl^- equilibrium potential E_{Cl} must be more negative than the resting potential and that intracellular chloride activity $(a_{Cl})_i$ must be lower than would arise from a purely passive distribution of Cl^- across the cell membrane.

Measurements of $(a_{Cl})_i$ in molluscan neurones using Cl^- sensitive micro-electrodes (Kerkut & Meech, 1966; Oomura, Ooyama & Sawada, 1968) confirmed that in cells that were hyperpolarized by ACh $(a_{Cl})_i$ was low. Certain cells were found, however, in which $(a_{Cl})_i$ was *higher* than would be expected from a passive distribution. These cells were depolarized by ACh, and Oomura *et al.* (1968) suggested that ACh increased the Cl^- permeability of both types of cell; the cells then being hyperpolarized or depolarized according to their value of $(a_{Cl})_i$. This idea has been used to explain the varying actions of ACh in the mammalian central nervous system (Eccles, 1969). However, Kerkut & Meech (1966) showed that in a snail neurone which had a high value of $(a_{Cl})_i$ the depolarization caused by ACh was probably due to an increase in Na^+ permeability.

When the original measurements of $(a_{Cl})_i$ were made it was customary to describe molluscan neurones as either 'H cells' or 'D cells' on the basis of their response to ACh, H cells being hyperpolarized by ACh and D cells being depolarized. Further work has shown that molluscan neurones can have more than one receptor for ACh, and that ACh can thus increase the permeability to more than one ion (Kehoe, 1972*a*, *b*). These recent findings show that H cells and D cells are by no means homogenous populations even with respect to the mechanism of their response to ACh. Gerschenfeld (1973) has reviewed the subject in detail.

The aim of the work described in this paper was to measure $(a_{Cl})_i$ directly with a new design of Cl^--sensitive micro-electrode and to see if there was any consistent relationship between $(a_{Cl})_i$ and the effects of ACh. The terms H cell and D cell are retained in this paper because the problem has always been framed thus, and it is the most convenient way to compare our work with previous work on the same subject. An H cell is defined simply as a cell that is hyperpolarized when ACh is applied to it at its normal membrane potential, and a D cell is defined as a cell that is depolarized when ACh is applied to it under the same conditions.

METHODS

Experiments were done on large neurones in the visceral and right pallial ganglia of the snail *Helix aspersa*. The ganglia were dissected and mounted in a small chamber in which they were continuously superfused with snail Ringer, as described by Thomas (1972).

The cells of the right pallial and visceral ganglia vary considerably in their properties, and it is important to be able to identify the cells being studied. The position and to a lesser extent the size of a given cell varies from preparation to preparation so that these criteria are inadequate to identify the cells, and no reliable maps of the ganglia have been published. Fig. 1 shows the usual positions of the cells studied. All the cells had a resting potential between 40 and 50 mV and fired action potentials spontaneously at the rate of one every 1 or 2 sec.

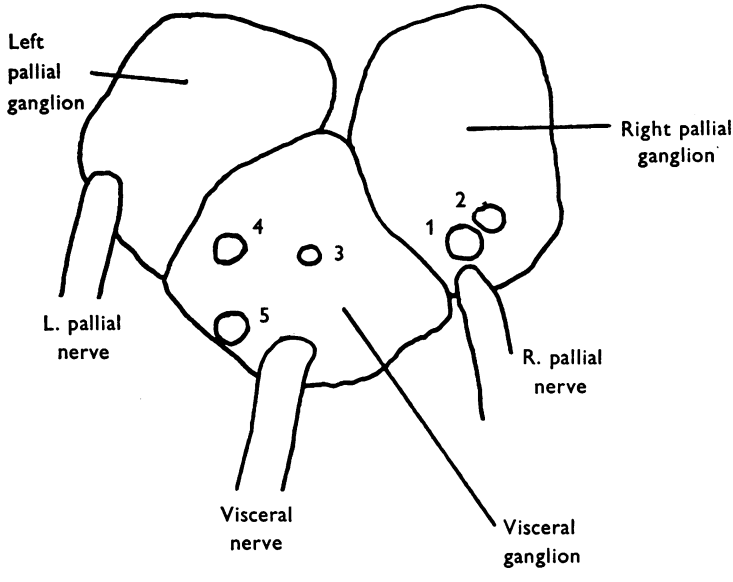

Fig. 1. Diagram of the dorsal surface of the suboesophageal ganglia of the snail *Helix aspersa* to show the usual positions of the cells studied.

Cell 1. This was the easiest cell to identify. It was 175–225 μm in diameter and was the largest cell in the right parietal ganglion. The duration of the action potential, not including the after-hyperpolarization, was 20–40 msec. The cell was depolarized by ACh, and showed an occasional 'inhibition of long duration', an inhibitory potential lasting 2–3 sec (Tauc, 1960). This cell has been studied by many other workers; it was cell A of Kerkut & Meech (1966), cell 7 of Kerkut, French & Walker (1970), cell 1 of Glaizner (1967), and the cell used by Thomas (1969).

Cell 2. This cell was 150–170 μm in diameter and had action potentials of 10 msec or less in duration. It often fired very rapidly for a few seconds after penetration. ACh depolarized the cell and usually decreased the height of the action potential, suggesting a large increase in membrane conductance. Identification of this cell was not absolutely certain, and there may have been two cells in this region of the ganglion with similar properties.

Cell 3. This was 100–125 μm in diameter and had a spontaneous synaptic input causing many 'fast i.p.s.p.s' (in the terminology of Gerschenfeld (1973)). It was hyperpolarized by ACh.

Cell 4. This cell was 150–175 μm in diameter, showed spontaneous fast i.p.s.p.s and was hyperpolarized by ACh. It probably corresponded to cell 3 of Kerkut *et al.* (1970), and was one of the cells studied by Kerkut & Thomas (1964). Its position in the visceral ganglion was quite variable and it is possible that there were two cells of similar size and properties.

Cell 5. This cell was 150–200 μm in diameter and had very similar properties to cell 1. It probably corresponds to cell 4 of Kerkut *et al.* (1970).

The normal snail Ringer had the following composition: NaCl 80 mM, KCl 4 mM, $CaCl_2$ 7 mM, $MgCl_2$ 5 mM, Tris maleate pH 8 20 mM. When the K concentration was changed complementary changes were made in the Na concentration.

Conventional glass micro-electrodes were made from borosilicate glass tubing as described by Thomas (1972). They were filled with 0·5 M-K_2SO_4 or 2 M-KCl when used to record membrane potential, or with 2 M-K acetate for passing current across the cell membrane, and had resistances between 15 and 25 MΩ.

Chloride-sensitive micro-electrodes. Recessed-tip Cl^--sensitive micro-electrodes of the type described by Neild & Thomas (1973) were used. They were calibrated using solutions of KCl for which the mean activity coefficients were taken from Parsons (1959), and gave a response of 56 mV for a tenfold change in Cl^- activity. The only physiologically occurring ions that interfered with their response to Cl^- were bromide and iodide. The speed of response of the electrodes depended on the tip diameter and the volume of the recess. The electrodes used here had tip diameters of 1–2 μm and responded completely to a rapid change in chloride activity within 2 min, with more than 90 % of the response occurring in the first minute.

Electrical arrangements. The membrane potential (E_m) was recorded as the difference between a conventional glass micro-electrode inside the cell and an indifferent electrode in the bath. The Cl^--sensitive micro-electrode registered both E_m and a voltage, V_{Cl}, that varied with chloride activity. To obtain V_{Cl} alone the signal from the membrane potential electrode was subtracted electrically from the signal from the Cl^--sensitive electrode. E_m and V_{Cl} were displayed on an oscilloscope and a multi-channel potentiometric pen recorder.

The membrane potential was controlled by passing current across the cell membrane through a second intracellular glass micro-electrode. The current was provided by either a current clamp or a simple voltage clamp circuit.

ACh was applied iontophoretically to the cell body using a double-barrel glass micro-electrode. One barrel was filled with 0·5 M-AChCl and the other with 2M-K acetate. A retaining current of about 5nA was passed continuously between the barrels, with the barrel containing ACh negative, to minimize ACh leakage. ACh was ejected by passing a current of about 300 nA in the opposite direction for 2–3 sec.

RESULTS

Fig. 2 shows the beginning of an experiment, with both the membrane potential electrode and the Cl^- sensitive electrode initially outside the cell. The Cl^--sensitive electrode was put into the cell first as it had the larger tip and caused the most movement of the cell. It recorded the membrane potential (E_m) and a potential V_{Cl} that depended on the intracellular Cl^- activity $(a_{Cl})_i$. The noise on the record is due to spontaneously

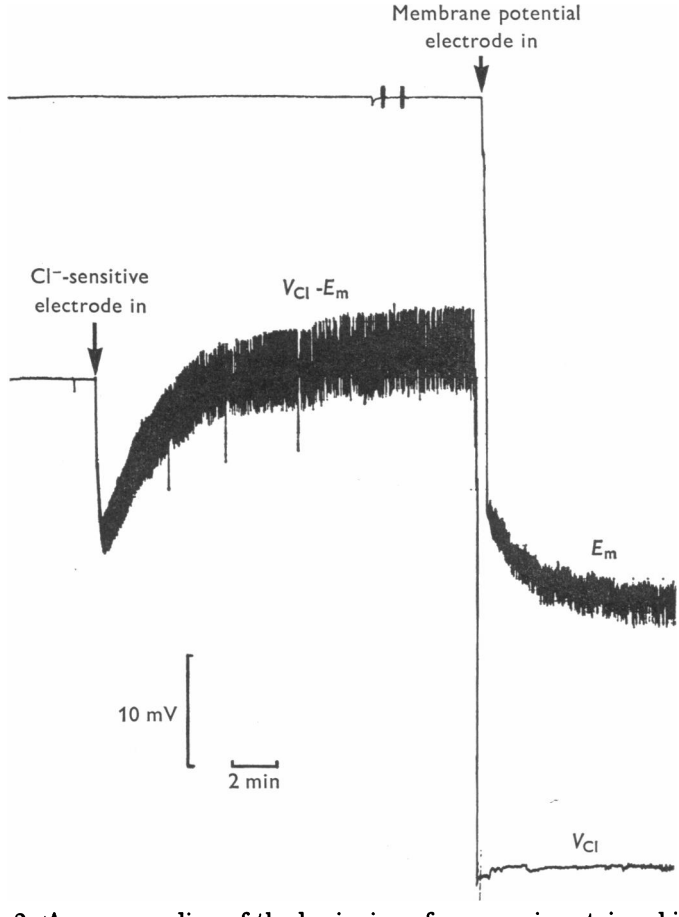

Fig. 2. A pen-recording of the beginning of an experiment in which $(a_{Cl})_i$ was measured. The upper trace shows the membrane potential recorded between a conventional glass micro-electrode and an indifferent electrode in the bath. The lower trace shows the signal between the Cl^--sensitive micro-electrode and the membrane potential recording electrode. The upper trace leads the lower trace by about 30 sec to prevent collision of the pens.

The Cl^--sensitive electrode was put into the cell first and it registered both the membrane potential (E_m) and a potential V_{Cl} that was proportional to the intracellular Cl^- activity ($(a_{Cl})_i$). An upward movement on the trace from this electrode could be caused by either a hyperpolarization of the cell or an increase in $(a_{Cl})_i$; the upward movement of the lower trace during the first few minutes after penetration represents the recovery of E_m after damage caused by the insertion of the electrode. The noise on the record is due to spontaneous action potentials that are greatly attenuated by the slow response of the pen-recorder.

After about 17 min the electrode to record membrane potential was inserted, and there was a downward deflexion of both traces. The upper trace was then a record of E_m and the lower trace became a record of V_{Cl} only; because E_m was subtraced electronically from the voltage from the Cl^--sensitive electrode.

occurring action potentials that were attenuated by the slow response of the pen recorder. When the membrane potential electrode was put into the cell only V_{Cl} was recorded on the trace from the Cl^--sensitive electrode because both electrodes were then recording E_m.

V_{Cl} would be equal to the Cl^- equilibrium potential E_{Cl} (but of opposite sign) if the slope of the calibration graph for the Cl^--sensitive electrode was equal to the theoretical value of $-RT/F$ (where R, T and F have their usual meanings), i.e. if the electrode gave a response of 58·7 mV per tenfold change in Cl^- activity at a room temperature of 22° C. In practice, a typical electrode gives only a -56 mV/decade response, thus in this case to find E_{Cl} it is necessary to multiply V_{Cl} by $58{\cdot}7/-56$. V_{Cl} can also be used to find $(a_{Cl})_i$ using the calibration graph of the electrode.

The recessed-tip chloride-sensitive micro-electrode was used to measure E_{Cl} and $(a_{Cl})_i$ in forty-one D cells and twenty-nine H cells. No difference was observed between the values from H and D cells, but the data are difficult to interpret because it was found that the Cl^- sensitive electrode changed its properties when inside the cell. The experiments which showed this are described in the next section. The apparent mean values of E_{Cl} were: in H cells $42{\cdot}2 \pm 1{\cdot}5$ mV (S.E. of mean, $n = 29$) and in D cells $42{\cdot}7 \pm 1{\cdot}1$ mV (S.E. of mean, $n = 41$). These values correspond to apparent intracellular chloride activities of 15·3 mM for H cells (95 % confidence limits 13·6 and 17·1 mM) and 15·0 mM for D cells (95 % confidence limits 13·8 and 16·3 mM).

The effects of ACh on H cells

The two H cells studied, cells 3 and 4, both had spontaneous i.p.s.p.s of the type that have been shown to be due to an increase in Cl^- permeability (Kerkut & Thomas, 1964; Chiarandini & Gerschenfeld, 1967). The same authors also showed that ACh increased the Cl^- permeability of the cells, and therefore the hyperpolarization caused by ACh must be accompanied by an influx of Cl^- ions into the cell and a rise in $(a_{Cl})_i$.

In some cases application of ACh to the cell body caused a rise in $(a_{Cl})_i$ large enough to be detected with the Cl^--sensitive micro-electrode. Such changes in $(a_{Cl})_i$ were usually too small to measure but could be made larger if the cell body was voltage clamped, because then the difference between E_m and E_{Cl}, which determined the movement of Cl^-, was not allowed to decrease. On two occasions it was possible in cell 4 to record a series of changes in $(a_{Cl})_i$ that were large enough to be measured easily. Part of the record from one of these experiments is shown in Fig. 3.

Fig. 4 is a graph of the change in $(a_{Cl})_i$, (which should be proportional to the number of Cl^- ions entering the cell assuming that the activity coefficient for Cl^- is the same inside as outside the cell), against the peak

current needed to prevent any change in E_m due to applications of a standard quantity of ACh at different values of E_m. The points are somewhat scattered because the changes in $(a_{Cl})_i$ are still too small to be measured very accurately. The least squares regression line through them passes almost exactly through the origin, showing that at the ACh reversal potential there was little or no change in $(a_{Cl})_i$. This indicates that in this cell ACh increases the membrane permeability to Cl^- ions only. The value

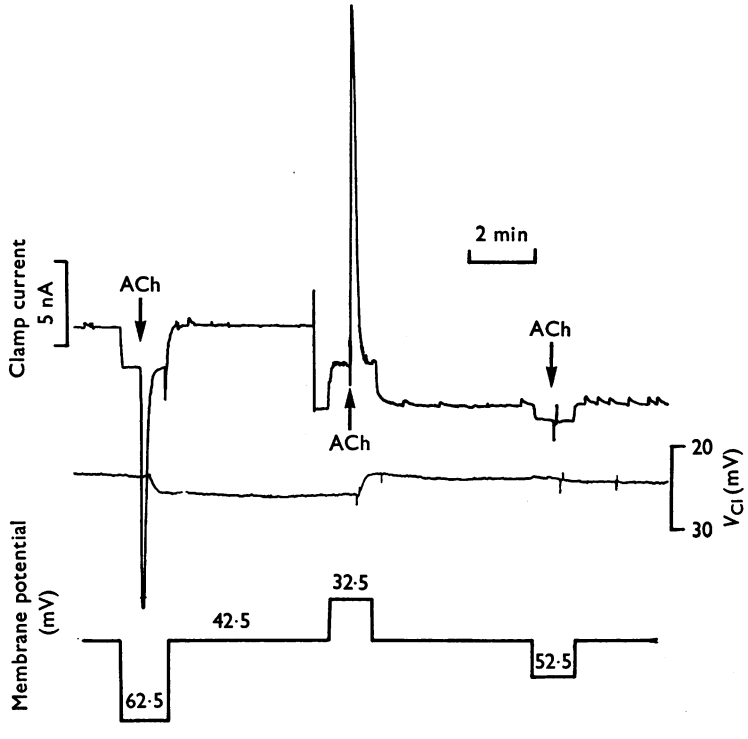

Fig. 3. Experimental record showing changes in clamp current (top trace) and intracellular Cl^- activity (middle trace) caused by iontophoretic application of ACh at different membrane potentials.

of E_m at which ACh caused no current to flow (E_{ACh}) was $-52{\cdot}5$ mV, and as ACh increased only the Cl^- permeability this was also the value of E_{Cl}. The estimate of E_{Cl} obtained from the Cl^--sensitive electrode at this point was only $-25{\cdot}4$ mV, an error of 27·1 mV.

In the other experiment where such an analysis was possible the results were similar. All the current was carried by Cl^- ions, and E_{ACh} was -53 mV, but the estimate of E_{ACh} obtained from the Cl^--sensitive electrode was only $-42{\cdot}5$ mV, an error of 10·5 mV.

The difference between E_{ACh} and the estimate of E_{Cl} made with the

Cl^--sensitive electrode was measured in a further twelve H cells. In five of the cells E_m was recorded using a micro-electrode filled with 2 M-KCl instead of 0·5 M-K_2SO_4, and in four of these the leakage of Cl^- from the electrode caused a large increase in $(a_{Cl})_i$. A recording from such an experiment is shown in Fig. 5. ACh was applied to the cell body at the arrows and at one minute intervals after the second arrow, each application

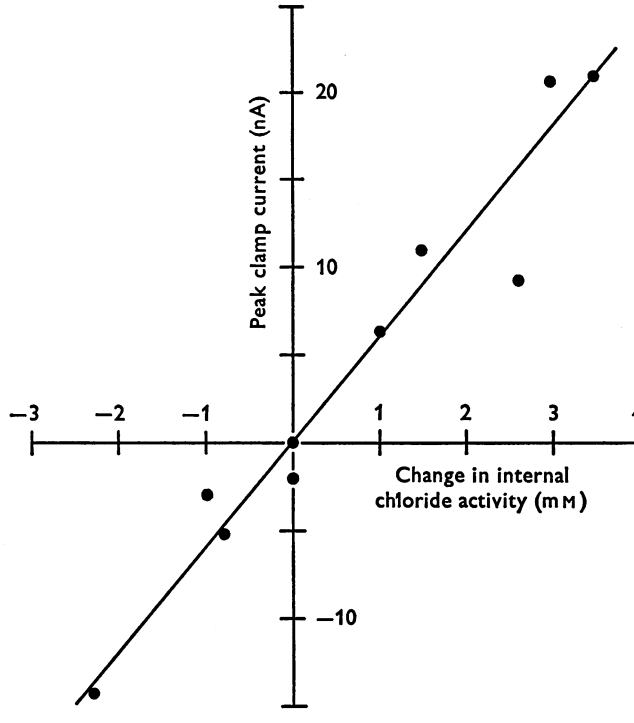

Fig. 4. Graph of the changes in $(a_{Cl})_i$ caused by the application of a standard quantity of ACh at various values of E_m, against the size of the peaks in clamp current in one cell. The line through the points is the least-squares regression line.

causing a small artifact on the record of V_{Cl}. At first ACh caused a hyperpolarization of the cell but this was soon changed to a depolarization as $(a_{Cl})_i$ rose. At one point ACh caused no change in E_m, showing that E_m was equal to E_{ACh}. The changes with respect to time in both V_{Cl} and the potential to which E_m moved when ACh was applied can be described by exponential curves fitted using the method of least squares. The time constants are −4·7 and −4·8 min respectively, and the correlation coefficient is greater than 0·99 in both cases.

In all fourteen H cells in which E_{ACh} and V_{Cl} were measured, the

estimate of E_{Cl} obtained with the Cl^--sensitive electrode was always less negative than E_{ACh}. Fig. 6 is a graph showing the relationship between the two quantities. There is an obvious correlation between them but it is equally obvious that the points are not scattered around a line of unit slope passing through the origin, as would be expected if the estimates of E_{Cl} provided by the Cl^--sensitive electrodes were correct. The mean value of E_{ACh} is $-61{\cdot}7$ mV, not including cells which were penetrated with KCl-filled micro-electrodes (open circles in Fig. 6).

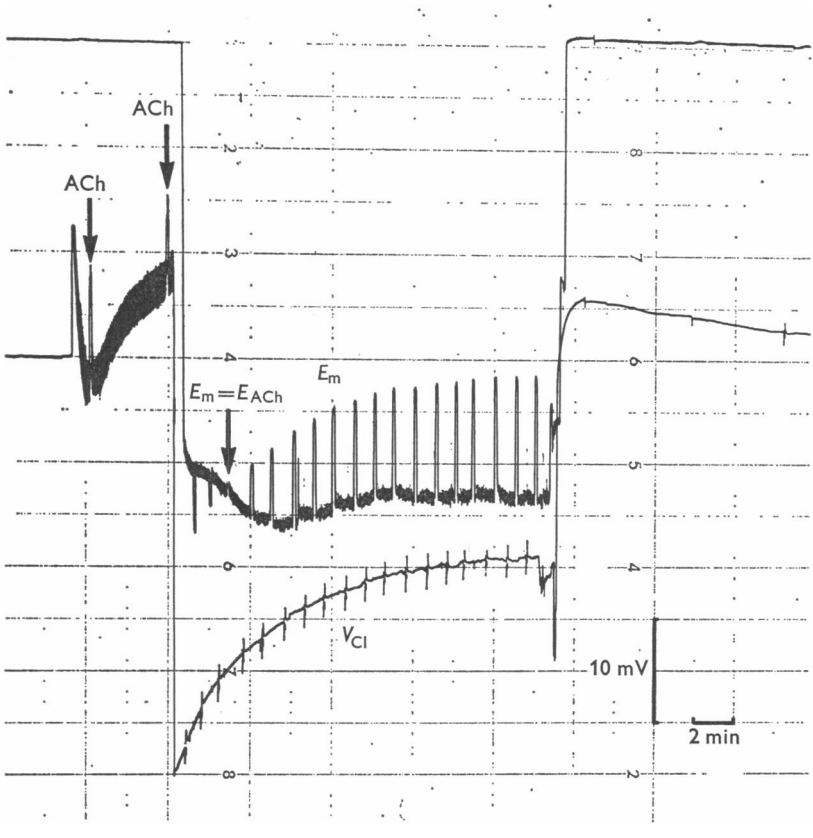

Fig. 5. Record from an experiment in which an electrode filled with 2 M-KCl was used to record E_m, showing a rise in intracellular Cl^- caused by leakage from the electrode. The electrode had a resistance of 22 MΩ. ACh was applied to the cell at the arrows and at 1 min intervals after the second arrow. Each application caused a small artefact on the record of V_{Cl}.

The mean error in the estimate of E_{Cl} using the Cl^--sensitive electrode was $15{\cdot}2 \pm 6{\cdot}2$ mV (S.D.). The best straight line through the points in Fig. 6 had a slope of 0·81, but this was not significantly different from 1. Although the size of the error varied from electrode to electrode, it was

found in experiments in which E_{ACh} was measured several times that the error remained constant throughout any one experiment. Thus this type of Cl^--sensitive electrode can be used to demonstrate changes in $(a_{Cl})_i$, but not to measure the absolute value of $(a_{Cl})_i$ directly.

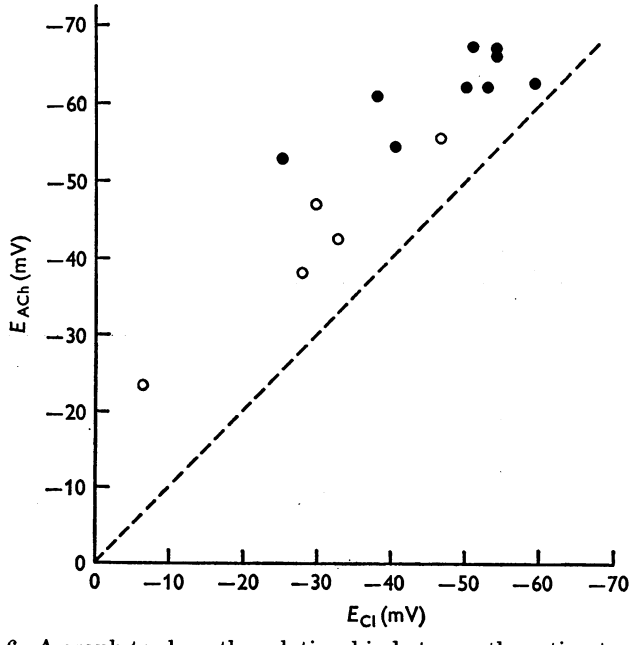

Fig. 6. A graph to show the relationship between the estimate of E_{Cl} made with the Cl^--sensitive micro-electrode and E_{ACh} in 14 H cells. The filled circles are from cells in which E_m was recorded with a micro-electrode filled with 0·5 M-K_2SO_4 and the open circles are from cells in which an electrode filled with 2 M-KCl was used. The dashed line through the origin is the line around which the points should be scattered if the electrode had been measuring E_{Cl} correctly.

The effects of ACh on D cells

The effects of ACh on $(a_{Cl})_i$ in D cells were different in different cells. In cells 1 and 5 ACh caused no detectable change in $(a_{Cl})_i$. E_{ACh} was not measured, but ACh still caused a depolarization when E_m was reduced to −20 mV. When 90 % of the Na in the extracellular solution was replaced by Tris the depolarization caused by ACh was greatly reduced. These findings are in agreement with those of previous workers on this type of cell (Chiarandini, Stefani & Gerschenfeld, 1967; Kerkut & Meech, 1966) and suggests that the ACh increases Na permeability.

In cell 2 ACh caused a depolarization followed by a slight hyperpolarization, and an increase in $(a_{Cl})_i$ (Fig. 7). There must have been an increase in Cl^- permeability, but it could not have been the cause of the

depolarization, since the net Cl^- flux was in the wrong direction. This suggested that the cell might have more than one ACh receptor and that ACh was increasing the permeability to both Cl^- and some other ion, presumably Na^+. It has previously been shown that the ACh receptor associated with an increase in Na^+ permeability is selectively blocked by hexamethonium (Tauc & Gerschenfeld, 1962; Kehoe, 1972*b*), and in this way multi-component responses to ACh can be analysed. When the cell

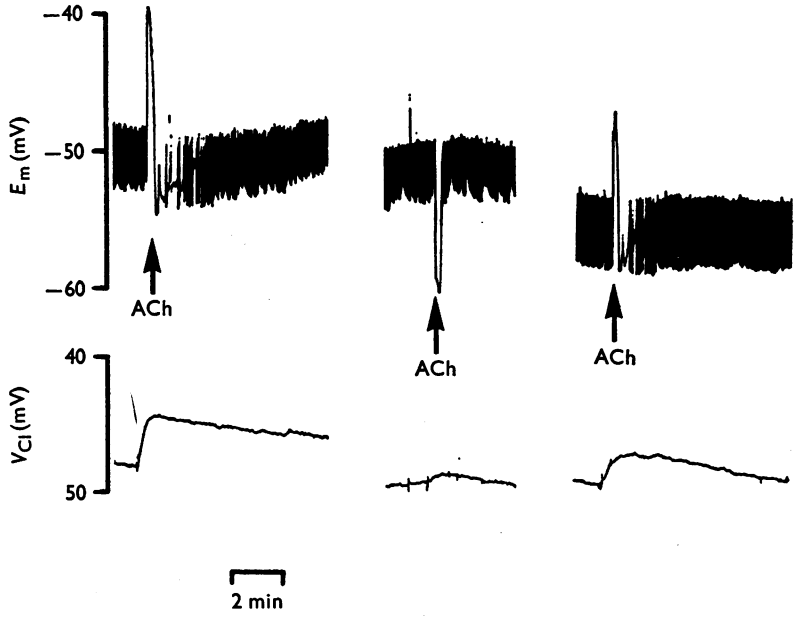

Fig. 7. Record showing the influx of Cl^- caused by ACh in cell 2. Left: ACh applied at the arrow caused a depolarization followed by a slight hyperpolarization, and a large increase in intracellular Cl^-. Centre: the same dose of ACh applied to the same cell in Ringer containing 2×10^{-4} M hexamethonium. Right: the response to ACh 10 min after washing off the hexamethonium.

was bathed in snail Ringer containing 2×10^{-4} M hexamethonium ACh caused a hyperpolarization, and there was still an influx of Cl^-. The Cl^- influx was smaller than before, probably because of the reduction of the difference between E_m and E_{Ch}. E_{ACl} in cell 2 in normal snail Ringer was between -35 and -45 mV, and in the presence of hexamethonium it changes to between -55 and -60 mV. When the cell was voltage-clamped it was found that there was no value of E_m at which ACh caused no extra current in the clamp circuit, and E_{ACh} was taken as the value of E_m when the current change was smallest. At this point the current was biphasic, but after hexamethonium no such biphasic current changes were

seen. Hexamethonium also reduced the size of the current change at any given value of E_m, as shown by the reduction in slope of the graph of E_m against peak clamp current (Fig. 8). It seems therefore that cell 2 has two independent receptors for ACh, one associated with an increase in Na^+ permeability and the other with an increase in Cl^- permeability.

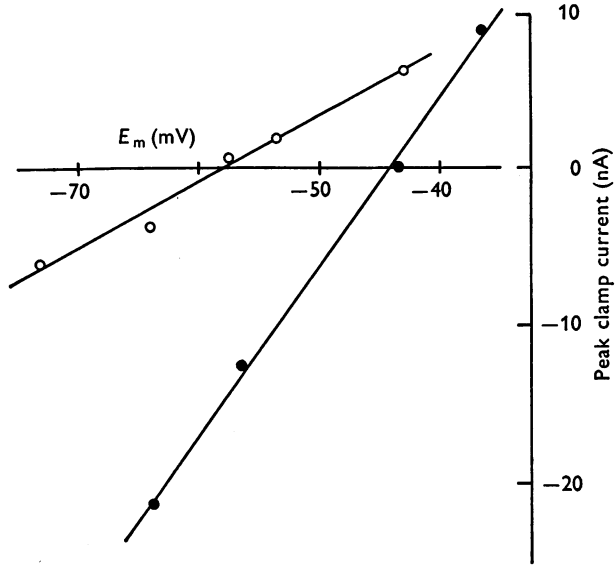

Fig. 8. Graphs of the size of the peaks in clamp current caused by ACh against E_m in cell 2. Filled circles: in normal snail Ringer. Open circles: in the presence of 2×10^{-4} M hexamethonium.

DISCUSSION

The properties of Ag/AgCl electrodes used intracellularly

The experiments on H cells showed that the estimate of E_{Cl} obtained with the Cl^--sensitive micro-electrode was always less negative than the true value of E_{Cl}, and that this error was probably constant throughout any one experiment. Such an error could have arisen from the presence in the cell of an ion such as Br^- or I^- which interfered with the response of the electrode to Cl^-, or some change in the standard potential of the electrode due to the different chemical conditions inside the cell. When there was sufficient data from an experiment it was possible to show that the error did not have the characteristics of interference from another ion (or ions), but that it seemed to be a simple electrical offset added to the electrode potential.

The potential E of an ion sensitive electrode can be described by the equation

$$E = E_o + S \log_{10}(a_m + K_{mn} a_n^{z_m/z_n}), \quad (1)$$

where E_c is a constant which has to be calculated for each electrode;

S is a constant, in practice taken as the slope of the calibration graph (for an ideal electrode it would be equal to 2·303 RT/F where R, T and F have their usual meanings and the factor 2·303 is used to convert from natural logarithms to logarithms to the base 10);
a_m is the activity of the ion to be measured;
a_n is the activity of the interfering ion;
z_m and z_n are the charges of the ions m and n;
K_{mn} is the selectivity constant for the ion n with respect to m (Hinke, 1961; Walker, 1971).

Using this equation it is possible to calculate new calibration graphs for the Cl^--sensitive micro-electrode which take into account the error that occurs inside the cell.

Fig. 9 shows such graphs for the electrode used in the experiment from which part of the data is shown in Fig. 3, and in which the electrode error was found to be 27 mV. (1) is the calibration line for the electrode obtained using KCl solutions as described in the methods section. (2) is the line that should be used if the electrode had a constant error of 25 mV added to its potential, thus shifting the line to the left but having no effect on the slope. (3) is the curve calculated assuming the electrode was subject to interference from one or more ions inside the cell. The difference

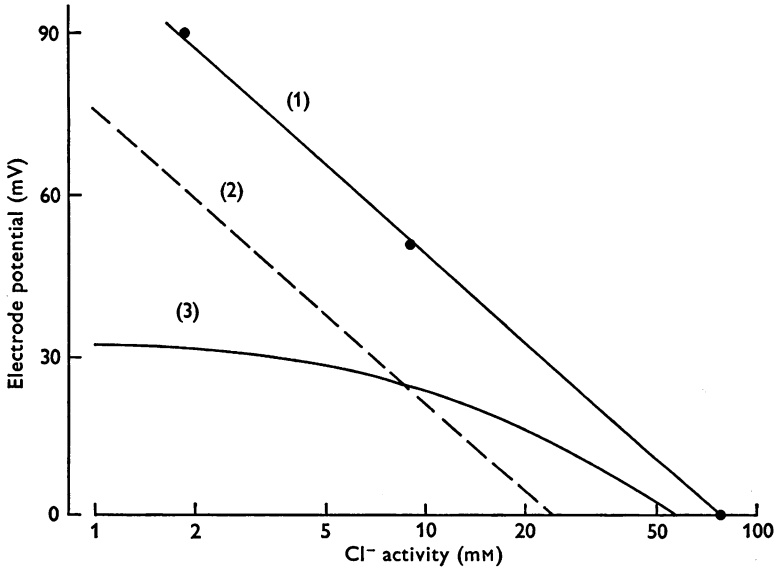

Fig. 9. Calibration graphs for a recessed-tip Cl^--sensitive micro-electrode. (1) Calibration line obtained experimentally using KCl solutions of known Cl^- activity. (2) Calculated intracellular calibration line assuming a constant offset voltage when the electrode is inside the cell. (3) Calculated intracellular calibration line assuming a constant activity of interfering ions inside the cell.

between the true value of $(a_{Cl})_i$ calculated from E_{ACh} and the value obtained with the Cl^--sensitive electrode was 19·1 mM, and this is equal to the term $K_{mn}a_n^{z_m/z_n}$ in eqn. (1), which can be used to calculate a new calibration curve for the electrode. In order to see which calibration curve was most nearly correct the total charge crossing the membrane after each pulse of ACh was calculated firstly from the current in the voltage clamp circuit and secondly from the change in $(a_{Cl})_i$, and the two values compared. To calculate the charge associated with the change in $(a_{Cl})_i$ the activities must be converted to concentrations by dividing them by the activity coefficient. Nothing is known about the activity coefficient for Cl^- in snail neurone intracellular fluid but from work on other preparations it seems reasonable to use the activity coefficient for the KCl solution of equivalent concentration (Keynes, 1963; Wallin, 1967), and a value of 0·77 was used. The volume of the cell was calculated from the diameter, which had been measured during the experiment, assuming that the cell was a sphere.

When the uncorrected calibration line ((1) in Fig. 9) for the electrode was used to calculate the charge entering the cell the values were about three times as large as those obtained from the changes in clamp current. This was as expected, as it was known that the Cl^- values used were erroneously high. When calibration line (2) was used the values of charge did not differ significantly from those calculated from the clamp current. When curve (3) was used the size of the chloride changes was quite similar to those obtained using the uncorrected line (1), although the absolute levels of Cl^- were lowered, in some cases to below 1 mM. The most valid correction therefore seems to be the one using line (2), i.e. the one that assumes that this electrode developed an offset of 27 mV when inside the cell. When the electrode was withdrawn from the cell its potential often did not return immediately to its previous extracellular level, but retained some offset, in the same direction as it had had inside the cell; this offset usually disappeared gradually over about 30 min. A similar phenomenon was noticed with another type of AgCl electrode by Hinke & Gayton (1971). Calibration lines for the electrode made during this recovery period had the same slope as that obtained before the experiment, showing that the electrode still had the expected sensitivity to changes in (a_{Cl}).

Although the results presented here are perhaps the first proof that AgCl electrodes do not respond as expected in an intracellular environment, several authors have raised objections to their use for measurements of $(a_{Cl})_i$ (Gesteland, Howland, Lettvin & Pitts, 1959; Tasaki & Singer, 1968; Janz & Ives, 1968). The arguments presented by all these authors are essentially the same. For an AgCl electrode to function correctly it must be surrounded by a saturated solution of AgCl, and so in solutions where there are no Ag^+ ions the electrode will continuously lose AgCl. This problem is not serious for large electrodes but miniaturised electrodes could, it is argued, lose most of their AgCl very quickly. This would be aggravated by the fact that the sulphydryl and amino groups of the proteins inside the cell form extremely stable complexes with Ag^+ ions, making it almost impossible to achieve an equilibrium between the AgCl and its surroundings.

Another reason suggested for the failure of AgCl electrodes is that the intracellular environment has sufficient reducing power to reduce AgCl to

metallic Ag. In support of this Gesteland *et al.* (1969) state that methylene blue is reduced intracellularly to its colourless form, and point out that in humans in cases of chronic silver poisoning metallic silver is deposited in the tissues. Janz & Ives (1968) also draw attention to the fact that substances such as acetone are adsorbed onto AgCl electrodes and change the electrode potential. Little is known about this effect and it is possible that some intracellular compound may do the same.

AgCl electrodes have been used to measure $(a_{Cl})_i$ in several types of cell (Mauro, 1954; Keynes, 1963; Strickholm & Wallin, 1965; Gayton & Hinke, 1968; Sato, Austin, Yai & Maruhashi, 1968; Oomura *et al.* 1968), but in only one case were the results seriously challenged. Strickholm & Wallin (1965) measured $(a_{Cl})_i$ in the crayfish giant axon with an electrode consisting of a fine chlorided silver wire protruding from the end of a glass capillary. They found $(a_{Cl})_i$ to be 25 mM, but Wallin (1967) later found a value of 12·7 mM by chemical analysis and suggested that the value obtained with the electrode was incorrect, perhaps due to a crack where the wire was sealed to the glass capillary. Assuming that the chemical analysis was correct, the error in their measurement of E_{Cl} with their AgCl electrode was 25 mV, quite similar to the average value of 15·2 mV found in the present experiments with the recessed-tip electrode. It seems likely that all Cl^--sensitive micro-electrodes that use AgCl as the sensitive element develop the same type of error in the intracellular environment and thus all give erroneously high values of $(a_{Cl})_i$.

The true value of $(a_{Cl})_i$ *in H and D cells*

The mean value of $(a_{Cl})_i$ recorded in H cells with the recessed-tip Cl^--sensitive micro-electrode was 15·3 mM, but it has been shown that this is larger than the true value. Using the fact that the mean difference between E_{ACh} and the apparent value of E_{Cl} was 15·2 mV the value of $(a_{Cl})_i$ can be corrected to 8·4 mM. This is close to the value of 8·6 mM found by Kerkut & Meech (1966) in their H cells. (Kerkut & Meech expressed their results as concentrations with the assumption that the activity coefficient for Cl^- was the same inside and outside the cell. Their values quoted here have been converted to activities using an activity coefficient of 0·77). Assuming that our error was the same in H and D cells, the corrected value of $(a_{Cl})_i$ in D cells is 8·2 mM, quite different from the value of 19·0 mM found by Kerkut & Meech. The discrepancy between the two results was not because they were from different cells. Kerkut & Meech used cell 1, and the values obtained here from cell 1 (twenty of the forty-one from D cells) were spread evenly throughout the range. Possibly our error was different in different types of cell, or the high value reported for cell 1 by Kerkut & Meech was due to the higher K content of their saline.

The most reasonable interpretation, however, of our results, obtained with the recessed-tip Cl^--sensitive electrode, is that in all the cells studied $(a_{Cl})_i$ is about 8·3 mM and therefore E_{Cl} is about -58 mV, a few millivolts more negative than the typical resting values of E_m. This view is supported by the effects of ACh on the cells.

The effects of ACh on H and D cells

The changes in $(a_{Cl})_i$ caused by ACh have not previously been reported in any preparation. The fact that in H cells $(a_{Cl})_i$ does not change when E_m is equal to E_{ACh} shows that ACh increases the cell membrane's permeability only to Cl^- ions. This is in agreement with results obtained by conventional methods in *Helix* and other molluscs (see Gerschenfeld (1973) for a review).

The results obtained from cell 2 are more interesting because there appear to be two types of ACh receptor on this cell. It is evident from the influx of Cl^- caused by ACh that ACh did increase the Cl^- permeability, but in order to depolarize the cell ACh must have increased the Na^+ permeability as well. The value of -35 to -45 mV for E_{ACh} in these cells is more negative than the value quoted for similar cells in Helix by Chiarandini *et al.* (1967), but their snail Ringer contained 120 mM Na whereas ours contained only 80 mM, and therefore the depolarizing effect of the Na component of the response would have been greater in their preparations.

The work of Ascher, Gerschenfeld & Kehoe (1972) has shown that there are cells in both *Helix* and *Aplysia* that have two ACh receptors, one of which when activated increases Cl^- permeability and the other of which increases Na permeability. The Na^+ receptor can be selectively blocked by hexamethonium leaving only the Cl^- component of the response. The effects of hexamethonium on cell 2 can be explained in the same way. With hexamethonium E_{ACh} was between -55 and -60 mV, the same as E_{Cl} in H cells and almost certainly the value of E_{Cl} in cell 2 also. E_{Cl} must certainly have been more negative than the normal resting potential of -50 mV because there was a small influx of Cl^- following an application of ACh even in the presence of hexamethonium (Fig. 7). It is possible, however, that application of a large amount of ACh to the cell for a prolonged period could lead to a large increase in $(a_{Cl})_i$ which would persist for some time. This may be one reason for the reports of high values of $(a_{Cl})_i$ in this type of cell.

The lack of any change in $(a_{Cl})_i$ caused by ACh in cells 1 and 5 is in complete agreement with the findings of previous workers that in these cells ACh increases only sodium permeability.

We wish to thank the Medical Research Council for financial support, and Dr P. Ascher for reading an early draft of the manuscript. T.O.N. was an S.R.C. Research Student.

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