J. Physiol. (1972), **225**, *pp.* 85–114
With 13 *text-figures*
Printed in Great Britain

IONIC MECHANISMS OF A TWO-COMPONENT CHOLINERGIC INHIBITION IN *APLYSIA* NEURONES

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(*Received 16 February 1972*)

SUMMARY

1. A two-component inhibition, consisting of a rapid and slow i.p.s.p., has been observed in the medial cells of the pleural ganglion of *Aplysia*. Each i.p.s.p. has been shown to be mediated by a distinct cholinergic receptor. The ionic mechanisms of the two components of the inhibitory response (whether elicited synaptically or by ACh injection) are analysed in this paper.

2. The inversion potential (typically -60 mV) of the rapid i.p.s.p. and of the rapid response to ACh injection is selectively altered by an intracellular injection of chloride or by partial substitution of the external chloride by impermeant anions. The shift caused by this last procedure is similar to that predicted for the chloride equilibrium potential (E_{Cl}) by the Nernst equation.

3. The slow i.p.s.p. and the slow response to ACh injection (both of which invert around -80 mV) are insensitive to changes in either internal or external chloride concentrations; on the contrary, with alterations of the concentration of potassium in the external medium, the inversion potential of the slow responses is altered in a way similar to that expected for the potassium equilibrium potential (E_K).

4. It is concluded that the rapid i.p.s.p. and the corresponding ACh potential are due to a change in chloride permeability of the post-synaptic membrane, whereas the slow responses are due to a selective change in potassium permeability.

5. Additional data suggest that the fast, 'chloride' channel is impermeable to sulphate and methylsulphate, but slightly permeable to propionate and isethionate. The slow, 'potassium' channel is impermeable to caesium ions, whereas its permeability to rubidium ions is half that to potassium.

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6. The potassium permeability of both the non-synaptic and synaptic membrane is markedly reduced by an intracellular injection of either tetraethylammonium (TEA) or caesium. These ions not only block the cholinergic potassium currents (whether inward or outward) but likewise block the potassium currents activated in the same cells by an iontophoretic injection of dopamine.

7. The potassium dependent synaptic potentials are also selectively affected by manipulations known to block the electrogenic sodium pump. In the presence of ouabain or in sea water in which sodium has been replaced by lithium, there is an apparent reduction of these potentials which was shown to be simply a reflexion of the movement of E_K towards a less polarized level. This shift in inversion potential was not seen for the potassium dependent response to ACh iontophoretic injection. These results are interpreted in terms of accumulation of potassium ions assumed to occur in the extracellular spaces of the neuropile, but not in the thoroughly dissected somatic region.

8. Cooling was shown to eliminate, selectively, the synaptic and ACh potential changes caused by an increase in potassium permeability.

INTRODUCTION

Many chemically mediated post-synaptic inhibitory potentials have been shown to be the result of an increase in the membrane permeability to potassium and/or chloride ions. For example, the conclusion drawn by Burgen & Terroux (1953) that a selective increase in potassium permeability underlies the inhibitory effect of acetylcholine (ACh) on the heart has been confirmed both by the tracer studies of Harris & Hutter (1956) and by the micro-electrode studies of Trautwein & Dudel (1958). Potassium dependent inhibitory potentials have also been observed in molluscan neurones (Gerschenfeld & Chiarandini, 1965). On the other hand, inhibition in crustacean muscle has been shown to be due to an increased chloride permeability (Boistel & Fatt, 1958; Takeuchi & Takeuchi, 1967; Motokizawa, Reuben & Grundfest, 1969), and a similar mechanism is involved in inhibition at many other neuromuscular junctions (see Ginsborg, 1967, for references) as well as in certain molluscan neurones (Chiarandini & Gerschenfeld, 1967; Kehoe, 1967, 1968; Sawada, 1969; Blankenship, Wachtel & Kandel, 1971).

Whereas in the above examples the inhibitory potentials seem to be due to a change in permeability to either potassium or chloride, in some preparations (certain molluscan neurones, Kerkut & Thomas, 1964; crayfish stretch receptor neurone, Edwards & Hagiwara, 1959; Hagiwara, Kusano & Saito, 1960; motoneurones, see Eccles, 1966; guinea-pig taenia coli,

Bülbring & Tomita, 1969), the transmitter has been assumed to increase both potassium and chloride permeabilities. In all of these cases, however, the experimental evidence has shown that one of the two ions plays the major role, and the data pointing to a contribution by the second ion are sometimes either indirect or insufficient. Nevertheless, it is currently considered a possibility that an i.p.s.p. may involve a change in permeability to both chloride and potassium ions.

In the *Aplysia* pleural ganglia, some neurones respond to the firing of a presynaptic cholinergic neurone and to an iontophoretic injection of ACh with a two-component inhibitory response, in which each component is mediated by a pharmacologically distinct receptor (Kehoe, 1972*a*). Since the two components of the inhibition differ not only in their time course, but also in their inversion potential, it seemed probable that different ionic mechanisms underlie the two components.

The experiments presented in this paper were designed (*a*) to determine these ionic mechanisms, (*b*) to evaluate the specificity of the ionic channels involved, (*c*) to measure the sensitivity of these channels to agents known to interfere with potassium permeability, and finally (*d*) to study the effects on these potentials of the blockade of the Na-K pump.

Brief reports of some of these data have appeared elsewhere (Kehoe, 1967, 1968, 1969; Kehoe & Ascher, 1970).

METHODS

Preparation. Unless specifically noted, all experiments were performed on the pleural ganglia of *Aplysia californica* (obtained from Dr R. Fay, Pacific Bio-Marine Supply Co.). The perioesophageal ring of ganglia was isolated with the associated nerves and connectives. Either the entire ring was left intact, or one of the pleuropedal pair of ganglia was cut away and was pinned to the bottom of a Perspex or glass chamber lined with a silicone resin (Elastomere, RTV 141, Rhône-Poulenc.). The connective tissue was removed from the dorsal surface of the pleural ganglion which was oriented as shown in Fig. 1 of the second paper in this series (Kehoe, 1972*a*). For an identification of the neurones used in these experiments, see the same Figure.

Electrodes and electrical measurements. Double barrelled micro-electrodes pulled on a de Fonbrune microforge were used in all experiments. The electrodes were filled (unless otherwise noted) with 0·6 M-K_2SO_4, and their resistance ranged between 5 and 30 MΩ. One barrel was used for recording in conjunction with a unity gain electrometer (Filmorex), a Tektronix oscilloscope, and a Brush Mark 280 rectilinear pen recorder. In a limited number of experiments, a Devices M2 recorder replaced the Brush recorder. Records of ACh potentials were always taken from the pen recorder, as were those of most of the synaptic potentials. Measurements of the amplitude or shape of the action potential were made on photographic records of the oscilloscope trace upon the rare occasions when the action potential itself was studied. For the records of action potentials taken from the pen recorder, no voltage calibration is presented.

The second barrel of the double barrelled micro-electrode was used for controlling the level of the membrane potential and for estimating changes in the membrane resistance. This was done by passing either inward or outward current through this barrel. Contact with ground was made through an agar–sea-water bridge placed in the bath solution, and connected with a calomel or AgCl electrode.

In order to reduce any shifts in junction potentials during experiments, all electrodes were permitted to stabilize at least 1 hr before an experiment was begun. However, since minor shifts could still occur, membrane potential measurements were always verified later in the experiment by removing the electrode from the cell.

In some experiments, when it was particularly useful to have a larger than normal synaptic input, tetraethylammonium bromide (TEA 10^{-2} M) was used to replace K_2SO_4 in one of the barrels of the electrode used in the presynaptic neurone. Leakage or injection of TEA into this neurone caused an increase in the duration of the presynaptic spike which in turn caused an increase in transmitter release and an amplified synaptic potential (Kehoé, 1972*a*). Use of such as electrode is noted in the appropriate Figure legend.

Iontophoretic injection of drugs. Micro-electrodes pulled with the de Fonbrune microforge were used for making iontophoretic injections of approximately 0·1 M aqueous solutions of ACh chloride and other drugs (noted when used). Weak braking currents were constantly applied to avoid leakage from the micropipettes. The constancy of the injection current was controlled, and its value was empirically chosen for a given preparation in terms of the response amplitude it produced. Typically, 200–300 msec pulses of 100–600 nA were used for ACh injections.

Temperature. Unless otherwise specified, experiments were performed at room temperature.

Manipulation of internal and external concentrations of ions. The internal chloride concentration (in the post-synaptic cells) was increased by hyperpolarizing the cell through one KCl-filled barrel of a double-barrelled intracellular electrode. TEA ions, on the other hand, were injected simultaneously with sulphate ions by means of an interbarrel injection from a double-barrelled K_2SO_4, TEA-Cl electrode.

Except when ionic mechanisms were being studied, the ganglia were bathed in natural sea water. In experiments involving changes in the external ionic concentrations, the artificial sea water used in control conditions had the composition of solution *A* (Table 1) in which the pH was adjusted to 7·8 with about 10 mM Tris HCl. Changes in the external K^+ concentrations from 0 to 20 mM were made by omitting or adding solid potassium chloride in solution *A* without compensating for the resulting changes in osmotic pressure.

Changes in external chloride ion concentration were obtained by substituting various anions for chloride ion. For propionate, isethionate, and methylsulphate, 'half-chloride' solutions were made by replacing 305 mM sodium chloride by an equimolar amount of the sodium salt of the substituting anion. In the early experiments in which sulphate was used as a substitute, solution *B* (Table 1) was used as the 'half-chloride' sea water. This solution, however, was made without trying to account for variations in either osmotic pressure or activity coefficients that were linked with the use of an asymmetrical electrolyte like Na_2SO_4. Solution *B* can be presumed to have a different osmotic pressure and a different ionic strength from solution *A*. To take into account these possible changes, the experiments were repeated using solution *C* as control sea water, and solution *D* as chloride-free sea water. These solutions were made by taking advantage of the measurements of osmotic pressure and the potassium ion activity coefficient (γ_K) by Baker, Hodgkin & Shaw (1962) in solutions of similar composition. Solution *C* is nearly identical to the artificial sea water of these authors, and solution *D* is calculated from solution *J* of

their Table 2. Thus, solution *C* and *D* should be isosmotic and γ_K should be near 0·68 in both cases. To prepare 'half-chloride' solutions, *C* and *D* were mixed in equal amounts, and it was assumed that the activity coefficient of chloride in the resulting solution was the same as in *C*. The results from these experiments were not different from those obtained using solution *B*, suggesting that in this solution the variation of osmotic pressure cancelled the reduction of the activity coefficient.

TABLE 1

	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Sucrose
A	10	480	10	50	610	—	—
B	10	480	10	50	305	152·5	152·5
C	10	526	20	30	636	—	—
D	10	77·6	—	—	—	43·8	900·0

No correction was attempted for the chloride activity coefficient in the other anion substitutions, although it was probably also modified. The mean activity coefficient of a 0·5 M sodium propionate solution is 10% higher than that of an equimolar sodium chloride solution (Robinson & Stokes, 1959). On the contrary, the methyl sulphate solutions probably have a lower activity coefficient than the chloride sea water (see Baker *et al.* 1962).

RESULTS

Effects of intracellular injection of chloride on the inversion potential of the two component inhibitory response

The response of the medial pleural cells to an ionophoretic injection of ACh at resting level (e.g. −45 mV) consists of a rapid hyperpolarization partially superimposed upon and followed by a more slowly developing hyperpolarizing wave. Such a two-component response can be seen in Fig. 1*A* at −40 mV. The inversion potential of the rapid component of the ACh response can best be identified as the level at which there is a long latency to a change in potential following the injection artifact (compare −60 mV, Fig. 1*A*, with records taken at any other level). Since the peak of the slow component of the response usually occurs well after the termination of the early, rapid potential, its inversion can be defined as the potential at which there is an immediate return of the membrane to its present value following the end of the early response. Under normal conditions this typically occurs at −80 mV. Compare the record in which the slow component is 'in equilibrium' (−80 mV, Fig. 1*A*) with the records in which the membrane continues to go further away from the present level (e.g. −40 mV) or remains above it (−100 mV) following the end of the rapid component.

When the recording and polarizing electrodes are filled with 0·6 M potassium sulphate, hyperpolarizing the cell to −100 mV for a half-hour period causes no change in either of the inversion potentials (see Fig. 1*A*).

On the other hand, if the cell is hyperpolarized with an electrode containing 3 M potassium chloride, a marked shift in the inversion potential of the rapid component is readily obtained with no noticeable concomitant change in the inversion potential of the slow one. As can be seen in Fig. 1 *B*, a 30 min hyperpolarization of the cell to −100 mV with a potassium chloride electrode caused a 20 mV shift in the inversion potential of the

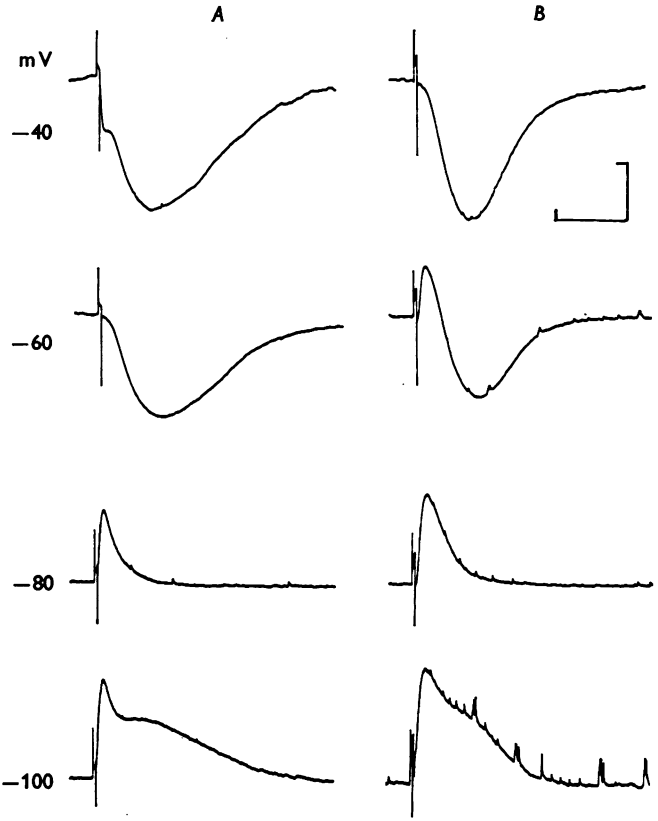

Fig. 1. Effects of intracellular injection of sulphate (*A*) and of chloride (*B*) ions on the inversion potentials of the two components of the ACh potential in the medial pleural neurones. *A*, the two-component inhibitory response following a 30 min hyperpolarization of the cell (to −100 mV) with an electrode filled with 0·6 M-K_2SO_4. No change was caused in either of the inversion potentials (−60 mV and −80 mV for the rapid and slow responses, respectively) by this hyperpolarization and the resulting injection of sulphate ions. *B*, the two-component inhibitory response following a 30 min hyperpolarization of the cell (to −100 mV) with a 3 M-KCl electrode. The resulting injection of chloride ions caused a movement of the inversion potential of the rapid phase from its normal −60 mV to −40 mV, whereas it had no effect on the inversion potential of the slow component, which remained at −80 mV. Calibration: 10 sec, 5 mV.

rapid phase (from -60 to -40 mV). Note that the -80 mV inversion potential of the slow component of the response was unchanged by the injection of either sulphate (Fig. 1*A*) or chloride (Fig. 1*B*) ions.

An intracellular injection of chloride has similar effects on the synaptic potential. For the identification of the inversion potential of the rapid component of the synaptic response, see the upper part of Fig. 2, which presents simultaneous recordings from the presynaptic neurone I (Pre) and the post-synaptic (medial pleural) neurone (Post). The membrane potential of the post-synaptic neurone in this recording was pre-set at approximately -50 mV. The presynaptic spikes early in the series evoked clearly hyperpolarizing i.p.s.p.s that diminish in size as the membrane reaches the potential of equilibrium of the rapid i.p.s.p.s (-60 mV). When equilibrium is reached, it is no longer possible to distinguish a rapid movement of the membrane potential (see arrow) following a presynaptic spike. However, since the slow i.p.s.p. is still hyperpolarizing at this level, it pulls the membrane almost immediately beyond the inversion point, and one sees rapid potentials reappear and increase in amplitude with further hyperpolarization. These rapid potentials are of course the inverted i.p.s.p.s which are associated, one for one, with the presynaptic spikes. The potential of inversion of the rapid i.p.s.p. is defined as the beginning of the approximatively 1 mV band where only the slow i.p.s.p. is readily visible, and this criterion is used in defining the equilibrium potential in all experiments under all changes in ionic concentrations.

Let us return now to observe the effects of intracellular injection of chloride on the inversion potential of the rapid i.p.s.p. In Fig. 2*A*, the slowly developing hyperpolarization without superimposed rapid potentials (i.e. the potential of inversion of the rapid i.p.s.p.) is seen at -60 mV. Following injection of chloride ions (Fig. 1*B*) this same type of record occurs at -50 mV, whereas at -60 mV the rapid potential is no longer in equilibrium and is markedly depolarizing. As was the case for the ACh potential, chloride injection had no noticeable effect on the inversion potential of the slow phase.

These data show that the rapid phase of the ACh and synaptic responses are chloride dependent, whereas the slow phases clearly are not. However, it is difficult to estimate the change occurring in internal chloride, and consequently a quantitative evaluation of these shifts in potential in terms of chloride concentrations cannot be made. Manipulation of external chloride concentration, on the other hand, allows such a quantitative evaluation of the change in the inversion potential of the rapid component and a comparison of these values with the changes anticipated by the Nernst equation for E_{Cl}.

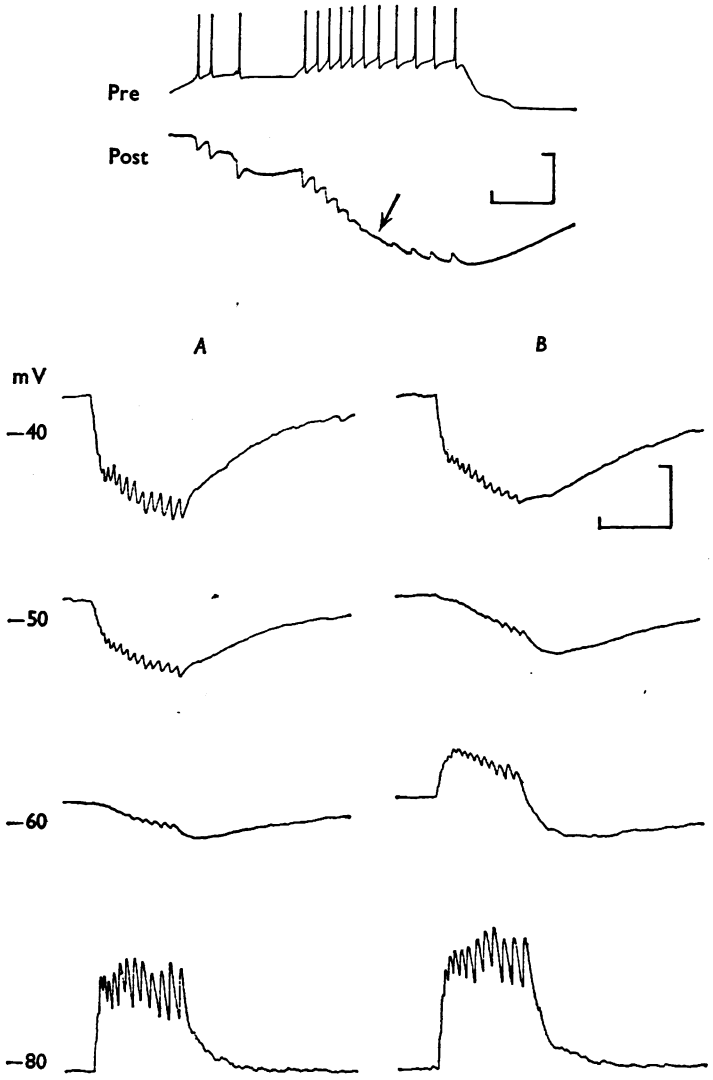

Fig. 2. For legend see opposite page.

Effects of changes in external chloride concentration

Effects of partial substitution of chloride by sulphate. Fig. 3 illustrates the effects on the ACh response of substitution of the external chloride by sulphate (see Methods). The inversion potential of the rapid response can be seen to occur at −60 mV in normal chloride solutions (Fig. 3*A*), at −53 mV in 75% chloride (Fig. 3*B*), and at −42 mV in 50% chloride (Fig. 3*C*). Note that sulphate, like methylsulphate (see below), causes a

reduction in the chloride-dependent response. However, this reduction is slight and does not impede an accurate estimation of the inversion potentials in the sulphate solutions. In Fig. 4, data from a number of experiments of this type are presented in graphic form, and it is shown that the values of the shift in inversion potential are essentially equal to those of the chloride equilibrium potential defined by the Nernst equation.

In these experiments, no noticeable change was observed in the inversion potential of the slow phase, and the original value of the inversion potential of the rapid phase was always recovered ($\pm$ 1 mV) with a return to the normal chloride concentration. It should be noted that the measurements were typically completed within a 10–15 min exposure to the thoroughly changed solution.

Comparable experiments performed on the synaptic input to the same post-synaptic cells yielded similar results. As can be seen in Fig. 5, when half of the chloride was substituted for by sulphate, the inversion potential of the rapid i.p.s.p. (which occurs at -60 mV in normal chloride solutions, see Fig. 5*A*) moves to -43 mV (Fig. 5*B*).

Once again, changes in external chloride had no apparent effect on the inversion potential of the slow phase. However, since it is difficult to measure the inversion potential of the slow i.p.s.p. when the rapid i.p.s.p.

Legend to Fig. 2.

Fig. 2. Effects of intracellular injection of sulphate (*A*) and chloride (*B*) ions on the inversion potentials of the two components of the cholinergic synaptic inhibition activated in the medial neurones by stimulation of presynaptic neurone I. The first two records in this Figure (Pre and Post) are included to facilitate the recognition of the inversion potential of the rapid phase of the two-component synaptic inhibitory potential. The post-synaptic response (Post) to a series of presynaptic (Pre) action potentials is recorded in a cell whose membrane was pre-set at approximately -50 mV. With repetitive firing of the presynaptic neurone, the initially hyperpolarizing IPSP associated with each presynaptic spike is reduced in amplitude, becomes imperceptible (see arrow) and eventually inverts as the summating slow i.p.s.p. pulls the post-synaptic membrane beyond the potential of inversion of the rapid i.p.s.p. The potential of inversion of the rapid i.p.s.p. is defined as the polarization level at which the rapid i.p.s.p. becomes imperceptible. Calibration: 1 sec, 5 mV.

A, the two-component inhibitory response following a 25 min hyperpolarization of the cell (to -100 mV) with an electrode filled with 0·6 M-K_2SO_4. No change was caused in -60 mV and -80 mV inversion potentials of the rapid and slow i.p.s.p.s respectively. *B*, a similar recording taken following the hyperpolarization of the same cell with an electrode filled with 3 M-KCl. The resulting injection of chloride ions caused a movement of the inversion potential of the rapid i.p.s.p. from -60 mV to -50 mV, whereas it had no effect on the inversion potential of the slow i.p.s.p. (-80 mV). Calibration: 1 sec, 5 mV.

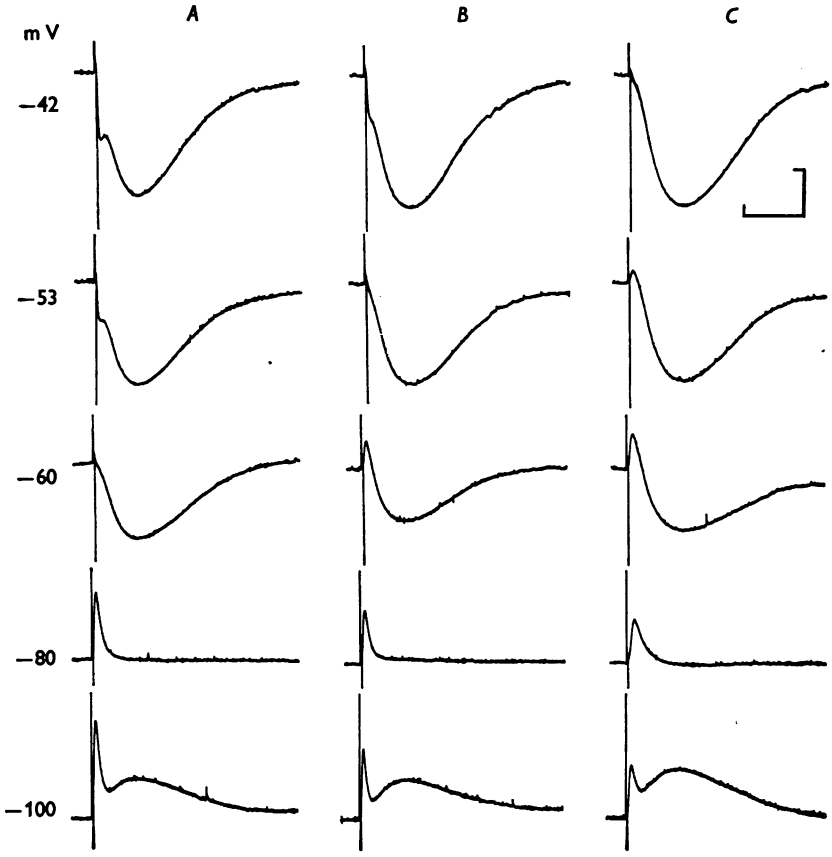

Fig. 3. Effects of a reduction of the extracellular chloride concentration on the inversion potentials of the two components of the response of a medial pleural neurone to iontophoretic injection of ACh. *A*: chloride sea water. *B*, *C*: replacement of 25% (*B*) and 50% (*C*) of chloride by sulphate. Note that under all conditions (*A*, *B*, *C*) the potential of inversion of the slow component remained at approximately −80 mV. The potential of inversion of the early, rapid component, on the other hand, moved from its normal −60 mV level (*A*) to −53 mV (*B*), and to −42 mV (*C*). Calibration: 10 sec, 5 mV.

is superimposed upon it, a more accurate estimate of the effects of changes in external chloride on the slow response was obtained after the rapid component had been blocked by tubo-curarine (see Kehoe, 1972*a*). No change in the potential of inversion of the slow i.p.s.p. was observed when half of the external chloride was substituted for by sulphate. Thus, one can conclude that the rapid inhibitory potential is due strictly to a movement of chloride ions, and that the slow i.p.s.p. does not involve a change in permeability to this ion.

Since the effects of changes in external chloride concentration on the rapid i.p.s.p. and on the rapid component of the ACh response are indistinguishable, the data gathered on the synaptic potential are included in the graphical presentation in Fig. 4.

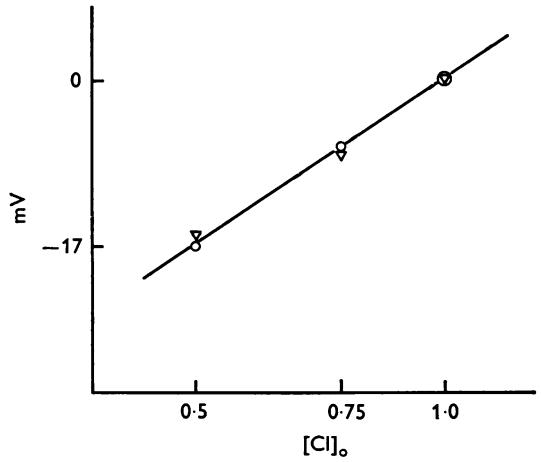

Fig. 4. Mean change in inversion potential of the rapid component of the cholinergic potential caused by reducing Cl_o by 25% (to 0·75 Cl_o) or half (0·50 Cl_o). Circles represent the mean of the values obtained for the rapid response to ACh injection (nine measurements at 1·0 Cl_o; five at 0·75 Cl_o; seven at 0·50 Cl_o). Triangles represent the mean of the values for the rapid synaptic response (two measurements at 1·0 Cl_o; one at 0·75 Cl_o; two at 0·50 Cl_o). The range never exceeded ± 1 mV around the mean value. The line represents the changes in E_{Cl} anticipated by the Nernst equation. The inversion potentials measured under 1·0 Cl_o, which varied only slightly around -60 mV, were arbitrarily assigned the 0 value from which all changes in inversion potentials were estimated. The line describing the anticipated changes in E_{Cl} was drawn through this 0 point. In approximately half of the experiments, chloride was replaced by methylsulphate; in the other half, by sodium sulphate.

Effects of substitution of chloride by methyl sulphate. Similar results to those obtained using sulphate in substitution for chloride were found when chloride was partially substituted for by methyl sulphate, although such experiments were complicated by an increased spontaneous activity stimulated by this foreign ion. Furthermore, the presence of methyl sulphate caused an almost immediate and very marked reduction in the chloride-dependent response. It was, nevertheless, possible to measure the inversion potentials when either 25 or 50% of the chloride was replaced by methyl sulphate, and the values obtained were indistinguishable from those found when sulphate was used as the substitute ion; thus, they are also included in Fig. 4.

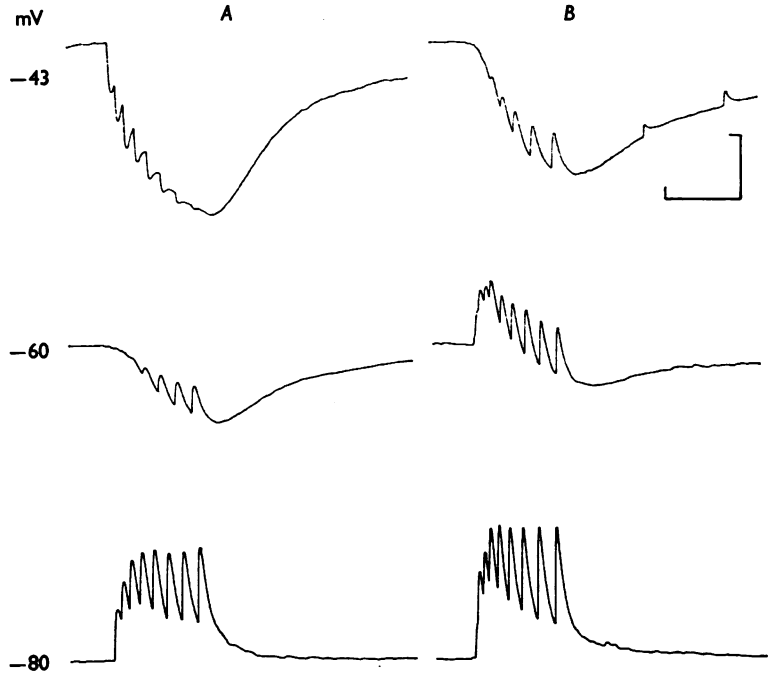

Fig. 5. Effects of a reduction by half of the extracellular chloride concentration on the inversion potentials of the two components of the synaptic inhibitory potential in a medial pleural neurone. Both in normal sea water (*A*) and after half of the chloride has been replaced by sulphate (*B*) the potential of inversion of the slow i.p.s.p., is approximately −80 mV. The potential of inversion of the rapid i.p.s.p., on the other hand, moved from the normal value of −60 mV (*A*) to −43 (*B*). Calibration: 2 sec, 5 mV.

Effects of substitution of chloride ions by propionate and isethionate. When half of the chloride was replaced by either propionate or isethionate, the movement of the inversion potential was considerably smaller than in the substitutions described above. Whether the effect is measured on the input or on the ACh potential, replacing 50% of the chloride by propionate causes only a 10 mV shift (from −60 to −50 mV) rather than the 17 mV shift which would be predicted from the Nernst equation if the membrane was completely impermeable to the substitute ion, and if chloride was the only ion involved in the potential.

Effects of changes in external potassium concentration

Effects of increasing or decreasing external potassium without substitution. The effects of changing the external potassium concentration on the ACh response can be observed in Fig. 6. The inversion potential of the slow

component (defined as the potential at which the membrane returns to and remains at the pre-set level immediately following the end of the rapid phase) occurs at −80 mV in 10 mM potassium (normal sea water) (see Fig. 6*B*). At other levels, the membrane potential only slowly returns to base line (e.g. at −96 mV in Fig. 6*B*) or it goes beyond the base line before finally returning to the pre-set level (e.g. at −70 mV, Fig. 6*B*).

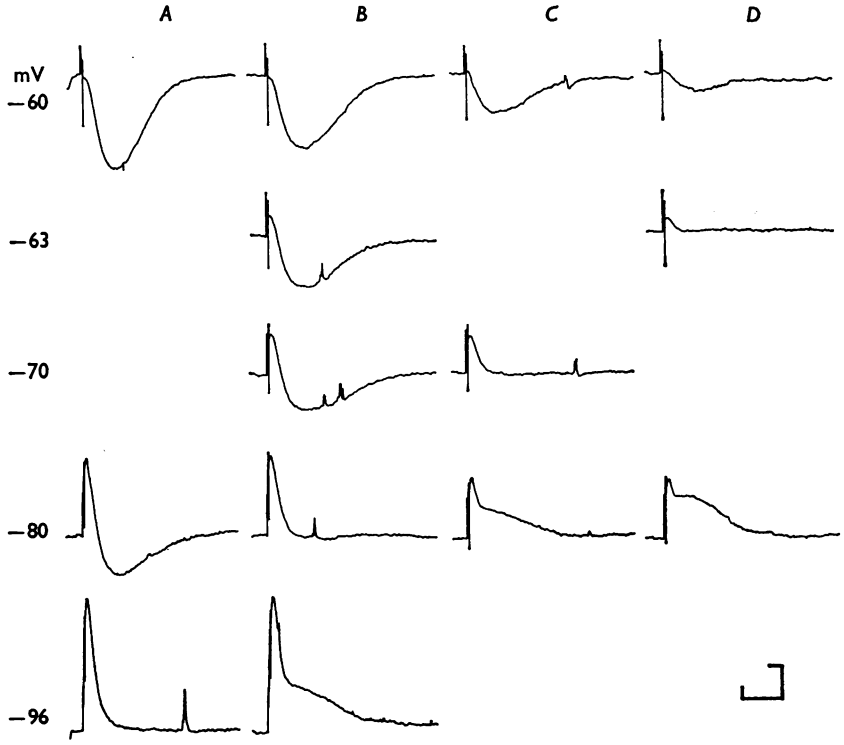

Fig. 6. Effects of alterations in the extracellular potassium concentration on the inversion potentials of the two components of the ACh response in a medial pleural neurone. Potassium concentrations, in mM: *A*, 5; *B*, 10; *C*, 15; *D*, 20. Going from half normal potassium (*A*) to twice the normal content (*D*) has no noticeable effect on the inversion potential of the rapid phase (see −60 mV). On the contrary, the potential of inversion of the slow component changes dramatically as a function of external potassium concentration (−96 mV in *A*, −80 mV in *B*, −70 mV in *C*, and −63 mV in *D*). Calibration: 10 sec, 5 mV.

When external potassium was halved (5 mM, Fig. 6*A*) the inversion potential was shifted to −96 mV. When potassium was increased to 15 mM (Fig. 6*C*), the inversion potential became 70 mV, whereas when potassium was doubled (20 mM, Fig. 6*D*), the inversion potential became −63 mV.

No shift in the −60 mV inversion potential of the rapid component was observed in the presence of varying concentrations of external potassium (see −60 mV at all concentration levels in Fig. 6).

In Fig. 7, data from a number of experiments of this type are presented in graphic form, and it can be seen that the shifts in inversion potential of

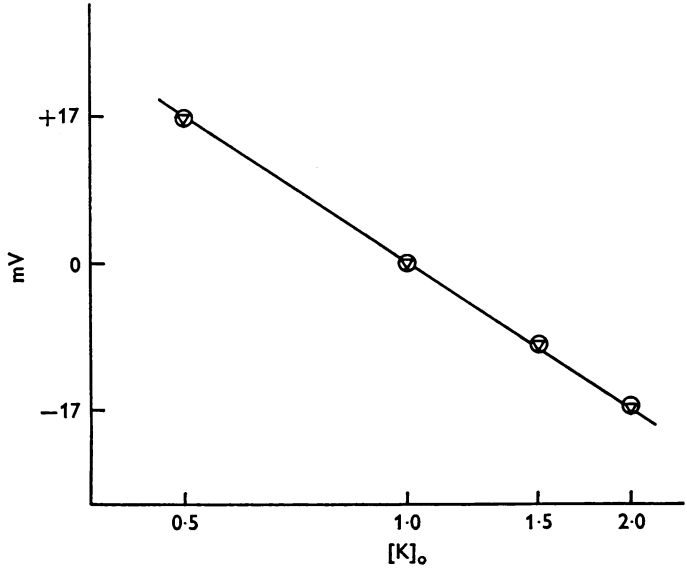

Fig. 7. Mean change in inversion potential of the slow component of the cholinergic potential caused by reducing K_o by half (0·5 K_o), by increasing it by half (1·5 K_o) or by doubling it (2·0 K_o). Circles represent the mean of the values obtained for the slow response to ACh injection (eight measurements at 1·0 K_o; eight at 0·5 K_o; seven at 1·5 K_o; six at 2·0 K_o). Triangles represent the mean of the values for the slow synaptic response (two measurements at 1·0 K_o; two at 0·5 K_o; one at 1·5 K_o; two at 2·0 K_o). The range never exceeded ±1 mV around the mean value. The line represents the changes in E_K anticipated by the Nernst equation. The inversion potentials measured under 1·0 K_o, which varied only slightly around −80 mV, were arbitrarily assigned the 0 value from which all changes in inversion potentials were estimated. The line describing the anticipated changes in E_K was drawn through this zero point.

All the experiments dealing with the synaptic response, and half of those concerned with the ACh potential, were performed on preparations in which the rapid phase had been pharmacologically eliminated.

the slow component are essentially equal to those of the potassium equilibrium potential defined by the Nernst equation. Measurements of the effects of changes in external potassium concentration on the ACh potential were usually terminated within 15 min following a thorough change of bathing medium, and original values were recovered when normal solutions were reinstated.

A similar picture was obtained when the synaptic potential was measured in different external potassium concentrations. Fig. 8 shows the effect of reduction of external potassium to half, and of doubling the normal concentration. Although it is difficult to observe the exact level of inversion potential of the slow i.p.s.p. when the rapid i.p.s.p. is superimposed upon it, one notices a marked movement in the inversion potential of the slow i.p.s.p., whereas there is no movement in the inversion potential of the rapid i.p.s.p. For example, if one observes the responses at -80 mV, it is clear that in 5 mM potassium (Fig. 8*A*) the membrane goes beyond the -80 mV pre-set level following the last inverted rapid i.p.s.p.; in 10 mM potassium (Fig. 8*B*), it returns to -80 mV following the last i.p.s.p. and

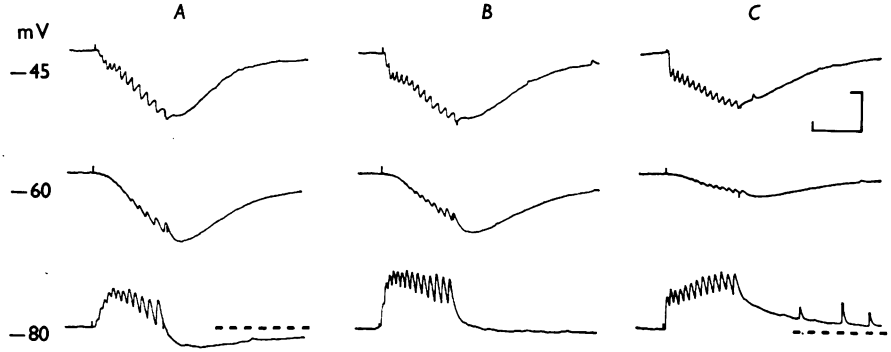

Fig. 8. Effects of alterations in the extracellular potassium concentration on the inversion potentials of the rapid and slow i.p.s.p.s in a medial pleural cell. Potassium concentrations, in mM: *A*: 5, *B*: 10, *C*: 20. The potential of inversion of the rapid i.p.s.p. remains at -60 mV whereas that of the slow i.p.s.p. varies (-97 mV in 5 mM; -80 mV in 10 mM; and -64 mV in 20 mV). Calibration: 1 sec, 5 mV.

remains there, whereas in 20 mM potassium (Fig. 8*C*) it returns to base line very slowly. By such criteria, the inversion potential of the slow i.p.s.p. appears to be approximately -64 mV in 20 mM potassium. The records taken at -60 mV under different external potassium concentrations show that the inversion potential of the rapid i.p.s.p. stays constant.

The effects of changes in external potassium concentration on the inversion potential of the slow i.p.s.p. are more accurately measured when the rapid i.p.s.p. has been pharmacologically eliminated (e.g. in a curarized preparation, see Kehoe, 1972*a*). As can be seen in Fig. 9, when the external potassium is halved (5 mM, Fig. 9*A*) the 'normal' -80 mV inversion level is shifted to -96 mV, whereas a shift to -63 mV is seen when the external potassium concentration is doubled (20 mM, Fig. 9*C*). When data from this and similar experiments are plotted in a semilog plot and compared with the changes predicted by the Nernst equation (see Fig. 7) it is found that

the movement of the inversion potential of the slow i.p.s.p., like that of the slow component of the ACh response, is that which would be predicted for a potassium electrode.

When measurements were made of the effects of changes in external potassium on the synaptic potential, a slightly longer period of exposure to the altered solution was required to obtain the 'final' value of the inversion potential than was necessary when the ACh potential was being studied. The slow i.p.s.p. inversion values used in the above figures were obtained approximately 10–15 min after initial exposure to the new potassium solution. Such a delay was not necessary for the establishment of the

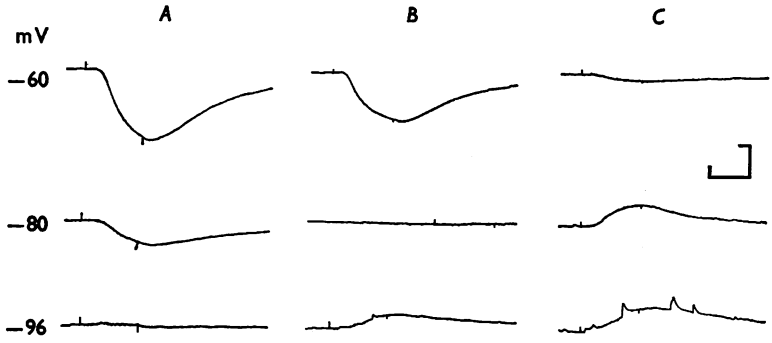

Fig. 9. Effects of alterations in the external potassium concentration on the inversion potential of the slow i.p.s.p. in a curarized preparation. The potential of inversion was found to be at −96 mV in 5 mM potassium (*A*), at −80 mV in 10 mM potassium (*B*), and at −63 mV in 20 mM potassium (*C*). Calibration: 1 sec, 5 mV.

maximum change in inversion level of the ACh potential. The ACh response is due to the activation of somatic receptors, which are in direct contact with the bathing medium. The synaptic response, on the other hand, is generated in the relatively inaccessible axonal region, which undoubtedly accounts for the lag in the establishment of the maximum shift in its inversion potential.

Effects of substitution of potassium by caesium ions. In a few experiments the effects of substitution of potassium by caesium were measured. As was shown in Figs. 6 and 7, reducing the normal potassium concentration (10 mM) to half (5 mM) causes a shift in the inversion potential of the slow component from −80 mV to approximately −97 mV. When the missing potassium ions were replaced by 5 mM caesium in such an experiment, there was no change in the −97 mV inversion potential. Thus, it appears that caesium cannot substitute for potassium in the development of the slow hyperpolarization. The effects of caesium substitution on the synaptic response were not measured.

Effects of substitution of potassium by rubidium ions. Rubidium, unlike caesium, does seem to serve as a partial substitute for potassium in the development of the slow component of the cholinergic potential. The addition of 10 mM rubidium to the normal bathing medium (containing 10 mM potassium) causes a shift in the potassium inversion potential of 10 mV, compared with the 17 mV shift typically occurring when the potassium concentration is doubled. Thus, the inversion potential in 10 mM potassium + 10 mM rubidium was found to be − 70 mV. When, on the other hand, the external solution contained 5 mM potassium + 5 mM rubidium, the inversion potential of the slow phase was − 86 mV.

These shifts of the slow phase inversion potential are those predicted by a ratio of rubidium and potassium permeabilities equal to 0·5 (see Discussion). However, additional observations suggest that they do not suffice to describe the effects of rubidium. When 10 mM rubidium is added to the normal sea water, one notes a rather marked reduction in the potassium dependent response which can only partially be explained by a change of membrane resistance. This reduction is accompanied by a noticeable shortening of the response which has no equivalent in an experiment where external potassium was increased to 15 mM or to 20 mM. This suggests that rubidium interacts with the movements of potassium (Adrian, 1964).

Effects of intracellularly injected TEA and caesium ions on potassium permeability

Both caesium and TEA ions are known to interfere with potassium permeability in a variety of preparations (see Hille, 1970, for review). When TEA is injected intracellularly into the medial pleural neurones, the action potential is markedly prolonged, presumably due to the elimination of the potassium outward current (see Fig. 10). 25 mM-TEA in the external medium, on the other hand, has no effect on the action potential. The effect of intracellular injection of TEA that is the most interesting, however, is the interference it causes with potassium movements involved in the synaptic and ACh potentials. As can be seen in Fig. 11, intracellularly applied TEA blocks the slow i.p.s.p. (Fig. 11*B*) as well as the slow component of the ACh response (Fig. 11*B'*) both of which have been shown to be potassium dependent. It is clearly seen in the records of Fig. 11*B'* that the block of the slow component is complete for both outward (see − 60 mV) and inward (see − 100 mV) currents. The dopamine response of these cells which has also been shown to be potassium dependent (Ascher, 1972) is likewise eliminated by intracellularly applied TEA.

Caesium ions have effects similar to those of TEA ions when injected intracellularly. Like TEA, they prolong the action potential and block both the inward and outward potassium currents triggered by synaptic and

iontophoretically applied ACh. Their effects on the response to dopamine injection were not studied.

A comparison of the effects of internal and external application of TEA and caesium is presented in the second paper of this series (Kehoe, 1972*a*).

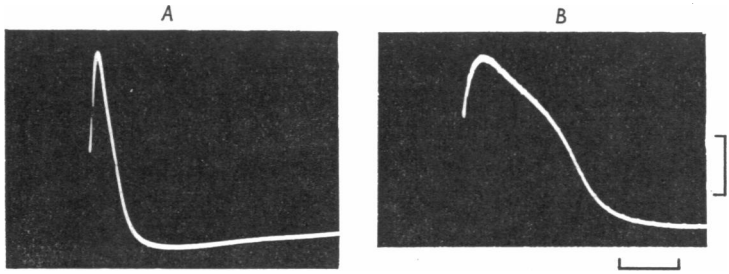

Fig. 10. Effects of TEA on the shape of the action potential of the medial pleural neurones. *A*, before injection of TEA. *B*, after intracellular injection of TEA and sulphate ions. Calibration: 20 msec, 20 mV.

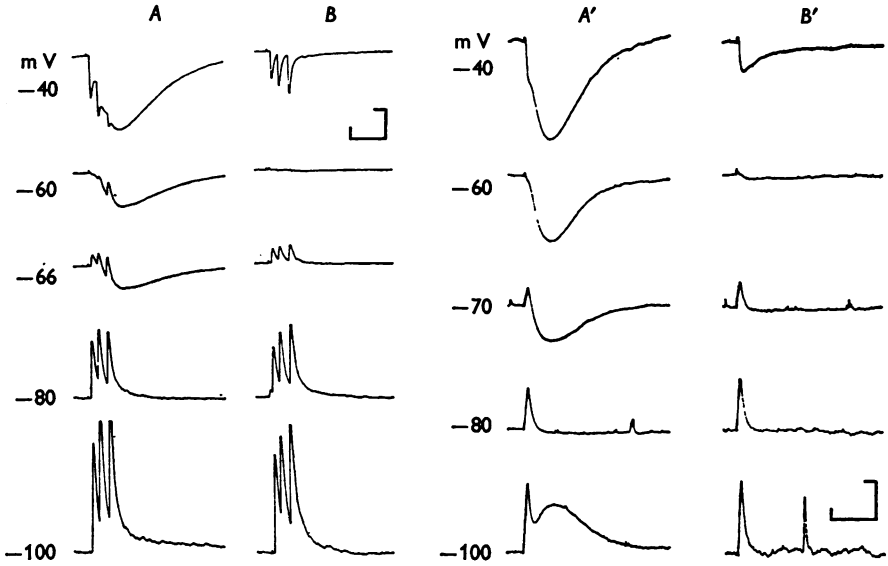

Fig. 11. Effect of intracellularly injected TEA on the potassium dependent potentials caused by cholinergic synaptic stimulation (*A*, *B*) or by iontophoretic application of ACh (*A'*, *B'*) in medial pleural neurones. Whereas the rapid component of these cholinergic responses is unaffected by the intracellular injection of TEA in the post-synaptic cell, the slow phase is completely eliminated. Calibration: *A*, *B*: 1 sec, 5 mV; *A'B'*: 10 sec, 5 mV. Note that in *A*, *B* the response to stimulation of presynaptic neurone I had been experimentally increased by a presynaptic intracellular application of TEA.

Effects of ouabain on the potassium-dependent inhibitory potentials

As can be seen in Fig. 12*A*, *B*, a few minutes following application of ouabain (5×10^{-4} M) to the bathing medium, the potassium dependent response observed at -60 mV is markedly reduced (see Fig. 12*B*). The amplitude of the rapid response, on the other hand, appears to be unchanged (see -80 mV, Fig. 12*A vs. B*), and it still inverts at -60 mV. However, a closer evaluation of the effects of ouabain on the potassium dependent slow i.p.s.p. shows that in the presence of ouabain this potential is no longer in equilibrium at -80 mV, but has rather become a depolarizing slow wave at this level (see Fig. 12*B*). Thus, there is a movement of the reversal potential, and this shift accounts for the reduction of the response seen at -60 mV. These records should be compared with those of Fig. 8 (at -60 and -80 mV) where one can observe the effects of changes in external potassium concentration on the two-component synaptic inhibition. A clearer picture of this shift can be obtained following the elimination of the rapid i.p.s.p. with curare. As can be seen in Fig. 12*A' vs. B'*, a 14 min exposure to ouabain (5×10^{-4} M) caused a 10 mV shift in the inversion potential of the slow phase of the synaptic response. The time required for the movement of the equilibrium potential appears to depend upon the level at which the post-synaptic cell is held. An artificial hyperpolarization of the post-synaptic cell to -100 mV slows down the movement of the inversion potential, undoubtedly due to the braking action it exerts on the exit of potassium from the cell.

It is interesting to note that ouabain does not produce a similar effect on the ACh potential, even following a 40 min exposure (see Fig. 12*A''*, *B''*). Although the response amplitude is reduced (due to a reduction in membrane resistance which occurs with prolonged exposure to ouabain) there is no shift in the inversion potential of the slow component (see -80 mV). As will be seen in the discussion, this selective action of ouabain on the synaptic potential can be explained by assuming that potassium accumulates in the extracellular synaptic space, but not around the exposed somatic region.

In light of such an interpretation, the shift in E_K in the synaptic region can be expected to be more or less pronounced as a function of the degree to which the synaptic area is in direct contact with the bathing medium. The effect does appear, in fact, to be more pronounced at similar cholinergic synapses of the visceral ganglion (Pinsker & Kandel, 1969; Kehoe & Ascher, 1970), where the synaptic contacts appear, by electrophysiological data, to be considerably farther from the soma than are the comparable contacts of the pleural cells. The slow hyperpolarization observed by Pinsker & Kandel (1969) in the visceral cells was initially attributed by these authors to the cholinergic activation of an electrogenic sodium pump, in part because ouabain did appear to block the response. This apparent block has been shown, however (Kehoe & Ascher, 1970), to be due at least in part to a shift in E_K.

That potassium does readily accumulate in the synaptic regions of those cells is further suggested by the following experiments which were performed using the presynaptic neurone L 10 of the visceral ganglion and one of the follower cells from the group L 1–L 6 (see Kandel, Frazier, Waziri & Coggeshall, 1967). These neurones respond with a two-component cholinergic inhibition similar to that of the medial pleural cells, but having certain unique features (see Pinsker & Kandel, 1969; Kehoe & Ascher, 1970; Waziri, 1971; Kehoe, 1972*b*). When the rapid i.p.s.p. of this

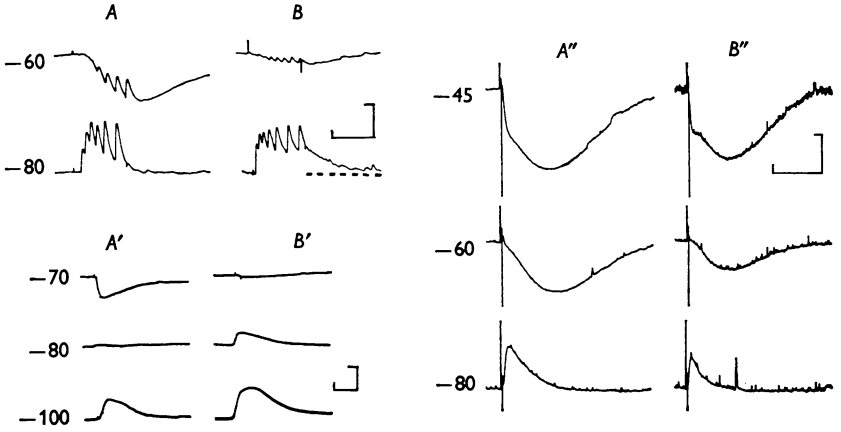

Fig. 12. Effects of ouabain (5×10^{-4} M) on the two-component cholinergic inhibition in the medial cells. *A*, *B*: the response of the medial cell to stimulation of presynaptic neurone I in normal sea water is shown in *A* at −60 mV (potential of inversion of the rapid i.p.s.p.) and at −80 mV (potential of inversion of the slow i.p.s.p.). When ouabain is added to the sea water, the slow i.p.s.p. appears to reduce in amplitude (see −60 mV, *B*). However, this reduction is merely a reflexion of the movement of the inversion potential of that response. The inverted slow i.p.s.p. can be identified at −80 mV, *B*, by the slow return of the membrane to the pre-set base line following the last i.p.s.p. (calibration: *A*, *B*: 1 sec, 5 mV).

A′B′: the movement of the inversion potential in the presence of ouabain is more clearly seen when the rapid phase has been blocked by curare (*A′*, *B′*). A 10 min exposure of the ganglion to ouabain is sufficient to cause a 10 mV shift in inversion potential (from −80 mV in *A′* to −70 mV in *B′*). Calibration: *A′*, *B′*: 2 sec, 5 mV.

A″, *B″*: on the other hand, no shift in the −80 mV inversion potential of the slow phase of the ACh response could be detected even after a 45 min exposure to ouabain. Calibration: 10 sec, 5 mV.

response is blocked by curare (as in Fig. 13 *A–C*) a slow hyperpolarization is unmasked that often can be separated into two elements: an 'early' hyperpolarizing wave that dominates during the firing of the presynaptic cell, and a 'later' component that follows the presynaptic firing (indicated by line above post-synaptic records). Both of these components have been shown to depend upon external potassium (Kehoe & Ascher, 1970), though the mechanism by which this potassium dependence occurs is likely different (see Discussion).

The earlier of these two curare-resistant responses appears to be inverted by polarization of the post-synaptic neurone, although this inversion often does not occur

until the soma is polarized beyond E_K (see Kehoe, 1972*b*). It has not been possible to invert the second component by somatic polarization, though it does 'disappear' when the soma reaches between -100 and -120 mV. As shown in the experiments below, however, both components can be inverted by repeated firing of the post-synaptic neurone. The experiment in the upper trace of Fig. 13 was performed on a cell in which the rapid i.p.s.p. of the two-component cholinergic response had been eliminated by curare, revealing the underlying slow components which were still hyperpolarizing at -85 mV. When, between records *A* and *B* (see arrow), the post-synaptic cell was fired 37 times and then returned to -85 mV, it was found that the

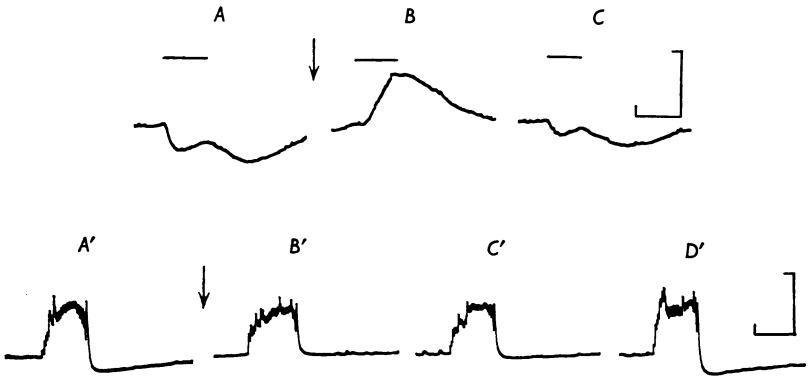

Fig. 13. Effects of previous firing of a post-synaptic visceral neurone on its synaptic response to stimulation of L 10. *A*, *B*, *C*: curarized preparation. The post-synaptic cell was first held at -85 mV, at which level the curare-resistant response is hyperpolarizing (*A*). The cell was then depolarized above firing threshold and fired 37 times (see arrow), and brought immediately back to -85 mV (*B*). All elements of the response to stimulation of the presynaptic neurone L 10 (indicated by the trace above the recording) were inverted following the period of post-synaptic action potentials. The synaptic potential did regain its normal hyperpolarizing form a few minutes following this post-synaptic spike activity (*C*). Record *C* was taken approximately 4 min following the firing of the post-synaptic cell.

A'–D': similar experiment performed in an uncurarized preparation, where the inverted rapid i.p.s.p. remains. Under normal conditions (*A'*) the rapid i.p.s.p.s are followed, even at -90 mV, by a hyperpolarizing phase. This phase is no longer visible following repeated firing of the post-synaptic neurone (arrow = 17 spikes). The response in *B'* was taken immediately following a return to the pre-set base line; that in *C'*, 4 min later; that in *D'*, 17 min later. Calibration: 5 sec, 5 mV.

response was clearly inverted and did not return to normal polarity until a few minutes later. Record *C* was taken 4 min following the firing of the post-synaptic cell.

In the lower trace (Fig. 13 *A'–D'*) are four records taken from an uncurarized preparation. The cell, held at -80 mV, responds to presynaptic firing (not shown) with a burst of inverted, rapid i.p.s.p.s followed by a slow, still hyperpolarizing i.p.s.p. Between records *A'* and *B'*, the post-synaptic cell was fired 17 times (see arrow) and then brought immediately back to the pre-firing level (-80 mV). When the response

to L10 was then tested, the late i.p.s.p. was no longer hyperpolarizing (Fig. 13*B'*) and only became slightly so after a 4 min pause (Fig. 13*C'*) and did not return to its original form until 15 min later (Fig. 13*D'*).

Effects of replacement of sodium by lithium on the potassium-dependent potential

When sodium is replaced by lithium in the sea-water bathing the ganglion, there is a shift similar to that caused by ouabain in the inversion potential of the potassium dependent synaptic response in the pleural cells. Unlike the effects seen under ouabain, however, the effects of sodium replacement by lithium are reversible. Moreover, when only half of the sodium is replaced, the effect is approximately half of that obtained with 100% replacement of sodium by lithium; i.e. there is only a shift of 4–5 mV. The effects of these sodium–lithium solutions on the ACh potential were not measured.

The effect of temperature on the two-component inhibition

The potassium dependent phase of the two-component inhibition, whether synaptically evoked or induced by ACh injection, is selectively eliminated by cooling the preparation to approximately 8° C. Cooling also causes an increase in duration of the presynaptic spike and a prolongation of the chloride dependent synaptic response (rapid i.p.s.p.). There is relatively little prolongation of the chloride dependent phase of the ACh response, suggesting that the effect observed on the synaptic chloride-dependent i.p.s.p. is due to a presynaptic change in the liberation kinetics (see Katz & Miledi, 1965).

DISCUSSION

Ionic mechanisms of post-synaptic inhibition

The data presented in this and the second article of this series (Kehoe, 1972*a*) show that the cholinergic inhibitory response seen in the medial cells of the pleural ganglion of *Aplysia* consists of two descriptively distinct components (one rapid, one slow) which are mediated by pharmacologically distinct receptors. The fact that changes in the intra- or extracellular chloride concentrations affect only the inversion potential of the rapid phase of the response, whereas the manipulation of external potassium affects only that of the slow component, indicates that each phase is due to the movement of a different ion. It becomes clear from such data that the fact that a synaptic potential of a given cell, or the response to artificial application of a supposed transmitter, is altered by changes in the concentrations of more than one ion does not necessarily imply a lack of selectivity in permeability change caused by a transmitter-receptor interaction.

Inhibitory potentials in a variety of preparations (certain molluscan neurones, Kerkut & Thomas, 1964; crayfish stretch receptor neurone, Edwards & Hagiwara, 1959, Hagiwara *et al.* 1960; motoneurones, see Eccles, 1966; guinea-pig taenia coli, Bülbring & Tomita, 1969) have been assumed to be the result of the simultaneous movement of both chloride and potassium ions, caused, presumably, by the interaction of the transmitter with a single receptor. In view of the data presented here from *Aplysia* neurones, it would seem justified to take a second look at certain of the preparations for which a bi-ionic mechanism has been proposed to see if some of the inhibitory responses are not in fact the result of the coincident activation of two receptors, each mediating a specific ionic permeability change. Although the two component nature of the inhibitory response of the medial cells is usually quite evident, this is not always the case. In certain *Aplysia* (*Aplysia fasciata*) the potassium dependent component is barely visible, and would not be noticed without a very careful analysis at different membrane potentials and confirmation by pharmacological separation of the two receptor-based elements (Kehoe, 1972*b*). Consequently, if the response of the medial pleural cells had been analysed only in *A. fasciata*, the complex nature of the inhibition might have been overlooked, and the response might have been thought to be a simple i.p.s.p. resulting from an unselective increase in permeability to both chloride and potassium ions, with chloride playing a predominant role.

As is shown in the last article of this series (Kehoe, 1972*b*), such a possible misunderstanding is not restricted to purely inhibitory potentials (see also Levitan & Tauc, 1972; Ascher, 1972).

Relationship between the inversion potential of the rapid component and the chloride equilibrium potential

That the inversion potential of the rapid component of the cholinergic inhibition is due to a selective increase in chloride permeability is supported by the shift observed after changing either external or internal chloride concentration, as well as by the absence of such shifts when the external potassium or sodium concentrations (unpublished observations) were modified.

The value of internal chloride concentration estimated by supposing that the chloride equilibrium potential and the inversion potential of the rapid component (-60 mV) are equal, could be expected to be approximately 53 mM. This value is similar to that calculated for cells of the visceral ganglion which are hyperpolarized by ACh (see Austin, Sato & Yai, 1967, 43 mM) and is also consistent with the chloride activity values measured by Kunze & Brown (1971) for similar cells in the same ganglion. Both groups of authors used chloride sensitive electrodes: the former designed

after those of Kerkut & Meech (1966); the latter using liquid ion exchangers.

The quantitative evaluation of the movement of the inversion potential of the rapid component with changes in external chloride is slightly restricted by the fact that most of the measurements were taken with solutions for which no measures of chloride activity are available, and in which chloride was substituted for by ions known to cause changes in chloride activity (see Methods). If we assume that in 50% chloride solutions in which chloride was substituted for by either sulphate or methylsulphate (see composition of solutions in Methods), the chloride activity coefficient is equal to that in normal sea water, it can be concluded that the inversion potential of the rapid phase does indeed change in accordance with the Nernst equation with changes in external chloride, thus behaving like an accurate chloride electrode.

Ionic selectivity of the synaptic chloride channel. That the Nernst equation accurately describes the shift in inversion potential of the rapid component when either sulphate or methyl sulphate are used as substitutes suggests that neither sulphate nor methyl sulphate can pass through the chloride channels opened by the transmitter. When, on the other hand, chloride was substituted for by either isethionate or propionate, the shift occurring in the inversion potential of the rapid component of the response was inferior to that predicted by the Nernst equation (10 *vs.* 17 mV change for a 50% reduction in chloride). These data suggest that the synaptic membrane is slightly permeable to these ions. However, caution must once more be exercised in the interpretation of these results, since no measure of chloride activity was made. The fact that the shifts were not identical for the four foreign anions used as chloride substitutes might be a manifestation, at least in part, of differences due to an incorrect evaluation of the chloride activity coefficients, leading either to an overestimation or an underestimation of the chloride activity (for example, isosmolar substitution of sodium chloride by sodium propionate solutions might lead to an underestimation of external chloride; sodium methyl sulphate, to an overestimation; see Robinson & Stokes, 1959).

Motokizawa, Reuben & Grundfest (1969) have used some of the same foreign anions to substitute for chloride in the analysis of the ionic mechanisms underlying the inhibitory junctional potential at the lobster muscle. It is interesting to note that their data suggest that isethionate passes less readily at this synaptic membrane than do methyl sulphate or propionate, whereas the data reported in this article suggest that the subsynaptic membrane of the medial pleural cells of *Aplysia*, on the contrary, is relatively permeable to isethionate as well as to propionate, but impermeable to methyl sulphate (or sulphate). These comparisons should

of course be re-evaluated in light of new data on the chloride activity of the various solutions employed.

Relationship between the inversion potential of the slow i.p.s.p. and the potassium equilibrium potential

Using for E_K the value of -80 mV (the inversion potential most frequently encountered for the slow phase of the synaptic and ACh potentials) the value of internal potassium predicted from the Nernst equation is 246 mM. This value corresponds favourably with that measured in the cells of the visceral ganglion by atomic energy absorption spectrophotometry (232 mM, Sato, Austin, Yai & Maruhashi, 1968) or by the use of potassium sensitive electrodes in the 'sister' cell of one of the neurones receiving input from presynaptic neurone I (236 mM calculated for right giant cell of visceral ganglion from the activity values obtained by Kunze, Walker & Brown, 1971).

Moreover, the movement of the inversion potential of the slow phase of the synaptic and ACh responses with changes in external potassium concentration (from 5 to 20 mM) is such that the predictions of the Nernst equation are fulfilled. Thus, this potential (like that studied by Trautwein & Dudel, 1958) appears to behave like that of an accurate potassium electrode.

Ionic selectivity of the synaptic potassium channel. The fact that the inversion potential of the slow component of the ACh or synaptic response is unaffected by addition of caesium to the external medium shows that caesium ions cannot replace potassium in the role of the generation of the slow inhibitory potential. Thus, the *Aplysia* synaptic membrane, like the excitable membrane of the squid giant axon (see, e.g. Pickard, Lettvin, Moore, Takata, Pooler & Bernstein, 1964) markedly discriminates between potassium and caesium ions.

The discrimination between potassium and rubidium ions, on the other hand, was not nearly so marked. In fact, as measured by the shift in the inversion potential, the synaptic membrane is approximately half as permeable to rubidium as it is to potassium ions. In 20 mM potassium the inversion potential is approximately -63 mV (as opposed to -80 mV in 10 mM potassium). When the external fluid contains 10 mM potassium and 10 mM rubidium, the inversion occurs at approximately -70 mV (the value typical for 15 mM potassium, 0 mM rubidium). The shift in inversion potential is adequately described by assuming that no rubidium has entered the cell at the time of the measurement and that the inversion potential is given by

$$E_{K,Rb} = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Rb}[Rb]_o}{P_K[K]_i}$$

with $P_{Rb}/P_K = 0{\cdot}5$, $[K]_i = 240$.

At the same time that rubidium ions share, in part at least, the ability of potassium ions to cause a reduced membrane resistance and to pass the synaptic membrane, they appear also to interfere with the movement of the potassium ions as suggested by the marked shortening of the slow wave which occurs in the presence of rubidium ions. In all of these respects, the permeability of this membrane to rubidium ions resembles that of frog muscle fibres (Adrian, 1964).

Effects of internal TEA and caesium ions on potassium permeability

TEA ions have been shown in a variety of preparations (see Hille, 1970 for review) to interfere with potassium permeability. The data presented in this paper show that TEA also interferes with potassium permeability in *Aplysia* neurones when applied intracellularly. Not only does it cause a prolongation of the action potential; it also blocks both inward and outward synaptic potassium currents, as well as those produced by ACh and dopamine injection. This blockade of the potassium permeability of the synaptic membrane is of particular interest, since the effectiveness of TEA against synaptic currents had not previously been demonstrated. The effects on potassium permeability of intracellularly applied caesium ions were qualitatively similar to those of TEA.

Effects of ouabain and removal of external sodium on the potassium equilibrium potential and the problem of potassium accumulation

That potassium liberated from a neurone during an action potential can cause a temporary accumulation of potassium in the restricted space just outside the membrane has been suggested by Frankenhaeuser & Hodgkin (1956) to explain changes in the undershoot of the action potential during successive impulses in squid axons, and by Orkand, Nicholls & Kuffler (1966) to explain a depolarization in the glial cells following action potentials in amphibian optic nerves. Using the undershoot of the action potential of leech neurones as a measure of E_K, Baylor & Nicholls (1969) estimated that a train of impulses could cause a temporary doubling of the normal concentration of potassium. This effect was abolished when the glial covering of the neurones was removed.

A similar potassium accumulation is hypothesized to explain the change in inversion potential of the potassium dependent synaptic response after the sodium–potassium pump has been blocked. That ouabain blocks the pump is well known. Replacement of external sodium by lithium ions appears to be an equally effective means of slowing the pump. The depletion of internal sodium which can be expected to result from eliminating that ion from the external fluid is sufficient to explain a marked slowing of the pump, particularly in view of the fact that lithium ions, even if they

do replace the internal sodium, are incapable of stimulating the sodium–potassium pump (Thomas, 1969).

When the pump is inactivated by either of these means, the potassium leaving the cell is no longer pumped back into the cell, and, in the event of diffusion barriers, it is quite reasonable to anticipate an accumulation of potassium ions in the extracellular space. This accumulation, unlike that caused by a finite series of action potentials, would not be expected to disappear with time since the source of excess potassium ions remains.

Affecting the pump rate by sodium replacement is of particular interest, since the effect is reversible, and apparently proportional to the amount of sodium removed from the solution (and/or the amount of lithium with which the sodium is replaced). This is suggested by the fact that the potential of inversion moves only about half as far in half-sodium solution as it does when all the sodium is replaced by lithium, or when ouabain is added to the bath.

It is interesting to note that no shift in the inversion potential of the slow component of the ACh response is seen even after a 40 min exposure to ouabain. The lack of effect on the somatic, ACh potential is presumably due to the fact that the region affected by the injection is in direct contact with the extracellular medium, and any potassium fleeing the somatic area (due to blockade of the pump) can diffuse readily into the bath. Thus, a change in E_K as measured by the somatic ACh potential will not occur until enough potassium has left the cell to alter significantly the internal concentration. Such a change apparently does not occur rapidly in the presence of ouabain (Kunze & Brown, 1971).

One other piece of data presented in this paper is also explained in terms of an accumulation of potassium in the extracellular synaptic area. As was shown in Fig. 13*A*, the repetitive firing of L10 causes, in some of the follower cells of the L1–L6 group in the visceral ganglion, two curare-resistant slow i.p.s.p.s: an 'early' component which dominates during the firing of the presynaptic cell, and a 'later' component which follows the presynaptic firing. The earlier of the two curare-resistant hyperpolarizations is cholinergic and is mediated by the same receptor type and via the same ionic mechanism (potassium ion permeability change) as is the slow i.p.s.p. in the medial pleural cells (Kehoe & Ascher, 1970). The latter of the two components is probably itself a mixture of the remaining part of the potassium-dependent cholinergic response and the hyperpolarizing phase of a biphasic response shown to be non-cholinergic, and presumed to be transmitted through an electrical synapse (see Kehoe, 1972*b*). The hyperpolarizing phase of this biphasic response, assumed to be the reflexion of a presynaptic 'post-firing' hyperpolarization, was shown (like the cholinergic post-synaptic slow wave) to increase with a halving of

external potassium (Kehoe & Ascher, 1970). Thus, its inversion following a train of post-synaptic spikes is assumed to be due to an augmentation of potassium in the juxta-synaptic region (see Fig. 13). Furthermore, this hyperpolarization was reduced, but not eliminated, in the presence of ouabain (Kehoe & Ascher, 1970), suggesting once more a shift in E_K similar to that seen for the pleural cells (see Fig. 12). The data thus suggest that this potential is the result of potassium-dependent 'after-potentials' in the presynaptic neurone. It must be considered that an electrogenic pump potential could contribute to the hypothetical *presynaptic* hyperpolarization. Such an interpretation is inconsistent, however, with the fact that the post-synaptic response increases when external potassium is halved, and persists (though altered) in ouabain. Furthermore, even if activation of the pump did contribute to such a presynaptic hyperpolarization, it is clear that the resulting post-synaptic response mediated by an electrical synapse could not be correctly considered the 'synaptic activation of an electrogenic pump' (see Pinsker & Kandel, 1970).

Possible misinterpretation of a potassium-dependent potential

Since ouabain, replacement of sodium by lithium, and cooling have all been shown to block the sodium–potassium pump, a hyperpolarizing potential that is eliminated by exposure to these three manipulations might readily be considered the result of activation of such a pump. However, the data presented here and in a previous note (Kehoe & Ascher, 1970) point out the prudence with which these criteria should be used. As was shown in Fig. 12, ouabain can give the impression of markedly reducing or eliminating a potassium-dependent potential. This reduction, however, is only apparent and is due rather to a movement of E_K, presumably in response to accumulation of potassium in extracellular spaces. Likewise, it was seen here that a potassium-dependent potential can also be eliminated very effectively by cooling, and thus the interpretation of the effects of temperature must also be very carefully made. If, in addition, a potential is electrotonic or is generated at a site distant from the point at which the recording is made, it may well be difficult to observe a conductance change (Diamond, 1968) or to invert the potential (Kehoe & Ascher, 1970; Kehoe, 1972*b*). Under such conditions, misinterpretation of the action of agents known to block the pump might occur even more readily.

This work was supported by grants to Dr Tauc from D.G.R.S.T., France (no. 70 02 164) and USPHS (NS 6975 04) and to Dr G. Horn from USPHS (NB 04787).

REFERENCES

ADRIAN, R. H. (1964). The rubidium and potassium permeability of frog muscle membrane. *J. Physiol.* **175**, 134–159.

ASCHER, P. (1972). Inhibitory and excitatory effects of dopamine on *Aplysia* neurones. *J. Physiol.* **225**, 173–209.

AUSTIN, G., SATO, M. & YAI, H. (1967). Osmolality effects in *Aplysia* neurones: I. Permeability coefficient and a model for water flux. *Math. Biosci.* **1**, 493–513.

BAKER, P. F., HODGKIN, A. L. & SHAW, T. I. (1962). Replacement of the axoplasm of giant nerve fibres with artificial solutions. *J. Physiol.* **164**, 330–354.

BAYLOR, D. A. & NICHOLLS, J. G. (1969). Changes in extracellular potassium concentration produced by neuronal activity in the central nervous system of the leech. *J. Physiol.* **203**, 555–569.

BLANKENSHIP, J. E., WACHTEL, H. & KANDEL, E. R. (1971). Ionic mechanisms of excitatory, inhibitory, and dual synaptic actions mediated by an identified interneuron in abdominal ganglion of *Aplysia*. *J. Neurophysiol.* **34**, 76–92.

BOISTEL, J. & FATT, P. (1958). Membrane permeability changes during inhibitory transmitter action in crustacean muscle. *J. Physiol.* **144**, 176–191.

BÜLBRING, E. & TOMITA, T. (1969). Increase of membrane conductance by adrenaline in the smooth muscle of guinea pig taenia coli. *Proc. R. Soc.* B **172**, 89–102.

BURGEN, A. S. V. & TERROUX, K. G. (1953). On the negative inotropic effect in the cat's auricle. *J. Physiol.* **120**, 449–464.

CHIARANDINI, D. J. & GERSCHENFELD, H. M. (1967). Ionic mechanism of cholinergic inhibition in molluscan neurones. *Science, N.Y.* **156**, 1955–1956.

DIAMOND, J. (1968). The activation and distribution of GABA and L-glutamate receptors of goldfish Mauthner neurones: an analysis of dendritic remote inhibition. *J. Physiol.* **194**, 669–723.

ECCLES, J. C. (1966). The ionic mechanisms of excitatory and inhibitory synaptic action. *Ann. N.Y. Acad. Sci.* **137**, 473–494.

EDWARDS, C. & HAGIWARA, S. (1959). Potassium ions and the inhibitory process in the crayfish stretch receptor. *J. gen. Physiol.* **43**, 315–321.

FRANKENHAEUSER, B. & HODGKIN, A. L. (1956). The after-effect of impulses in the giant nerve fibres of *Loligo*. *J. Physiol.* **131**, 341–376.

GERSCHENFELD, H. M. & CHIARANDINI, D. J. (1965). Ionic mechanism associated with non-cholinergic synaptic inhibition in molluscan neurones. *J. Neurophysiol.* **27**, 710–723.

GINSBORG, B. L. (1967). Ion movements in junctional transmission. *Pharmac. Rev.* **19**, 289–316.

HAGIWARA, S., KUSANO, K. & SAITO, S. (1960). Membrane changes in crayfish stretch receptor neurone during synaptic inhibition and under action of gamma-amino-butyric acid. *J. Neurophysiol.* **23**, 505–515.

HARRIS, E. J. & HUTTER, O. F. (1956). The action of acetylcholine on the movements of potassium ions in the sinus venosus of the heart. *J. Physiol.* **133**, 58–59*P*.

HILLE, B. (1970). Ionic channels in nerve membranes. In *Progress in Biophysics*, vol. 21, pp. 3–32, ed. BUTLER, J. A. V. & NOBLE, D. Oxford: Pergamon.

KANDEL, E. R., FRAZIER, W. T., WAZIRI, R. & COGGESHALL, R. E. (1967). Direct and common connections among identified neurones in *Aplysia*. *J. Neurophysiol.* **30**, 1352–1376.

KATZ, B. & MILEDI, R. (1965). The effect of temperature on the synaptic delay at the neuromuscular junction. *J. Physiol.* **181**, 656–670.

KEHOE, J. S. (1967). Pharmacological characteristics and ionic bases of a two-component postsynaptic inhibition. *Nature, Lond.* **215**, 1503–1505.

KEHOE, J. S. (1968). Double inhibition de certains neurones d'*Aplysie*. *J. Physiol., Paris* **60**, 266.

KEHOE, J. S. (1969). Blocage sélectif par l'ion tétraéthylammonium d'une inhibition cholinergique résistant au curare. *C. r. hebd. Séanc. Acad. Sci. Paris* **269**, 111–114.

KEHOE, J. S. (1972*a*). Three acetylcholine receptors in *Aplysia* neurones. *J. Physiol.* **225**, 115–146.

KEHOE, J. S. (1972*b*). The physiological role of three acetylcholine receptors in synaptic transmission in *Aplysia*. *J. Physiol.* **225**, 147–172.

KEHOE, J. S. & ASCHER, P. (1970). Reevaluation of the synaptic activation of an electrogenic sodium pump. *Nature, Lond.* **225**, 820–823.

KERKUT, G. A. & MEECH, R. W. (1966). The internal chloride concentration of H and D cells in the snail brain. *Comp. Biochem. Physiol.* **19**, 819–832.

KERKUT, G. A. & THOMAS, R. C. (1964). The effect of anion injection and changes in the external potassium and chloride concentration on the reversal potentials of the IPSP and acetylcholine. *Comp. Biochem. Physiol.* **11**, 199–213.

KUNZE, D. & BROWN, A. M. (1971). Internal potassium and chloride activities and the effects of acetylcholine on identifiable *Aplysia* neurones. *Nature, New Biology* **229**, 229–232.

KUNZE, D., WALKER, J. L. & BROWN, H. M. (1971). Potassium and chloride activities in identifiable *Aplysia* neurones. *Fedn Proc.* **30**, 348.

LEVITAN, H. & TAUC, L. (1972). Acetylcholine receptors: topographic distribution and pharmacological properties of two receptor types on a single molluscan neurone. *J. Physiol.* **222**, 537–558.

MOTOKIZAWA, F., REUBEN, J. P. & GRUNDFEST, H. (1969). Ionic permeability of the inhibitory postsynaptic membrane of lobster muscle fibres. *J. gen. Physiol.* **54**, 437–461.

ORKAND, R. K., NICHOLLS, J. G. & KUFFLER, S. W. (1966). Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. *J. Neurophysiol.* **29**, 788–806.

PICKARD, W. F., LETTVIN, J. Y., MOORE, J. W., TAKATA, M., POOLER, J. & BERNSTEIN, T. (1964). Caesium ions do not pass the membrane of the giant axon. *Proc. natn. Acad. Sci. U.S.A.* **52**, 1177–1183.

PINSKER, H. & KANDEL, E. R. (1969). Synaptic activation of an electrogenic Na^+ pump. *Science, N.Y.* **163**, 931–935.

ROBINSON, R. A. & STOKES, R. H. (1959). *Electrolyte Solutions*. New York: Academic Press.

SATO, M., AUSTIN, G., YAI, H. & MARUHASHI, J. (1968). The ionic permeability changes during acetylcholine-induced responses of *Aplysia* ganglion cells. *J. gen. Physiol.* **51**, 321–345.

SAWADA, M. (1969). Ionic mechanisms of the activated subsynaptic membrane in Onchidium neurones. *Jap. J. physiol.* **31**, 491–504.

TAKEUCHI, A. & TAKEUCHI, N. (1967). Anion permeability of the inhibitory postsynaptic membrane of the crayfish neuromuscular junction. *J. Physiol.* **191**, 575–590.

THOMAS, R. C. (1969). Membrane current and intracellular sodium changes in a snail neurone during extrusion of injected sodium. *J. Physiol.* **201**, 495–514.

TRAUTWEIN, W. & DUDEL, J. (1958). Zum Mechanismus der Membranwirkung des Acetylcholin an der Herzmuskelfaser. *Pflügers Arch. ges. Physiol.* **266**, 324–334.

WAZIRI, R. (1971). Electronically coupled interneurones produce two types of inhibition in *Aplysia* neurones. *Nature, New Biology* **232**, 286–288.