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HYPERPOLARIZATION OF RABBIT SUPERIOR CERVICAL GANGLION CELLS DUE TO ACTIVITY OF AN ELECTROGENIC SODIUM PUMP

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1 The mechanisms underlying the hyperpolarization which follows depolarization of rabbit superior cervical ganglion cells by acetylcholine, have been investigated and compared with the mechanisms responsible for the hyperpolarizations induced by orthodromic stimulation of the ganglion.

2 The amplitude of the drug-induced hyperpolarization (after-hyperpolarization) was diminished when $[Na^+]_0$ and the duration of the preceding depolarization were reduced.

3 In K^+-free solutions, the amplitude of the after-hyperpolarization was often diminished and its rate of development was reduced. In 12.5 mM K^+-Krebs solutions, the amplitude and rate of development of the after-hyperpolarization were increased; the potential was still present when the resting potential was at or close to E_K.

4 Ouabain (10 μM) prevented or greatly diminished the after-hyperpolarization. The rates of onset and decay of the after-hyperpolarization were reduced in glucose-free solutions.

5 It is, therefore, concluded that the after-hypolarization is due to the activity of an electrogenic sodium pump.

6 The positive after-potential associated with the ganglionic action potential was increased in K^+-free solutions and diminished when the resting potential approached E_K, indicating that it is due to a period of increased K^+ conductance. In the presence of high concentrations of hexamethonium (276 μM), the P wave was not selectively depressed by ouabain and has been shown by other workers to be due to a mechanism not involving an increased potassium conductance. It is concluded, therefore, that the positive after-potential, the P wave and the after-hyperpolarization are due to different mechanisms.

Introduction

It has been shown by several workers (Pascoe, 1956; Brown, 1966a & b; Kosterlitz, Lees & Wallis, 1968, 1970a; Brown, Brownstein & Scholfield, 1969, 1972) that, after superior cervical ganglia from rabbit, rat or kitten have been depolarized by choline, acetylcholine or carbachol, removal of the depolarizing agent results in a hyperpolarization (after-hyperpolarization) of the ganglion cells.

Furthermore, orthodromic nerve stimulation of these ganglia may cause hyperpolarization of the ganglion cells; analysis by extracellular recording techniques points to at least two distinct mechanisms being involved. First, there is a large positive after-potential, which is the consequence of the conductance changes associated with the falling phase of the ganglionic action potential. Secondly, there may be a hyperpolarizing post-synaptic potential (slow i.p.s.p.), without detectable conductance change (Kobayashi & Libet, 1968; Libet & Kobayashi, 1969); this latter potential is commonly referred to as the P wave (Eccles & Libet, 1961) and is considered by them to be catecholamine-mediated (see Libet, 1970).

Conflicting explanations have been advanced for the hyperpolarization which follows the acetylcholine-induced depolarization of the ganglion. Pascoe (1956) suggested that this long-lasting hyperpolarization was the sum of the positive after-potentials of cells which had fired action potentials. Kosterlitz *et al.* (1968) proposed that it was unlikely to have the same ionic basis as the positive after-potential because, unlike the latter, the amplitude of the after-hyperpolarization was

not increased in K^+-free solutions. They also noted that the rate of development of the after-hyperpolarization was greatly reduced in K^+-free solutions, a finding consistent with active extrusion of sodium ions from ganglion cells. More recently, it has been suggested (Watson, 1972) that, in the rat, the after-hyperpolarization is due to the action of released catecholamines on ganglion cells.

From what is known of the ionic basis of hyperpolarizations in other mammalian tissues, it is necessary to test at least three possibilities, namely an increase in conductance of the membrane to K^+, an increase in conductance to Cl^- or to Cl^- and K^+ and, thirdly, an active extrusion of Na^+. The last mechanism, which would be expected to be linked to a metabolically-dependent carrier system, could be either truly electrogenic or only apparently electrogenic (Kerkut & York, 1971; Koketsu, 1971); in the first condition, the coupling ratio between Na^+ extrusion and K^+ re-entry would not be unity and, in the second, a diffusion barrier round the cells might permit an electrically-neutral pump working at a high rate to generate a potential if the concentration of K^+ within the barrier fell.

A preliminary account of the evidence that the after-hyperpolarization is due to the activity of an electrogenic Na pump was given in a demonstration to the British Pharmacological Society (Kosterlitz *et al.*, 1970a). Brown and his co-workers (Brown & Scholfield, 1970; Brown *et al.*, 1972) have reached similar conclusions on the basis of their work on the rat superior cervical ganglion.

Methods

Preparation

Superior cervical ganglia were removed from adult New Zealand White rabbits (2.7-4.3 kg) anaesthetized with urethane (1.25-1.5 g/kg) given intravenously as a 25% (w/v) solution. The ganglia were prepared for insertion into the sucrose-gap apparatus, in which the sucrose compartment was separated by membranes, and electrical recordings were made by the method previously described (Kosterlitz *et al.*, 1968). In some later preparations, the potential changes were also displayed on a potentiometric pen recorder (Servoscribe RE 511).

The sucrose-gap method was chosen because, unlike the 'moving-fluid' electrode technique (Pascoe, 1956), it permits direct records of potential changes to be made from the ganglion or, independently, from their postganglionic axons and also because the potentials are larger. Stable recordings may be made over periods of up to 5 h; diffusion potentials are minimized with the membrane version of the sucrose-gap technique because the interface between solutions is almost entirely within the tissue.

Solutions

All solutions were made up from glass-distilled water. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11; it was gassed with 5% CO_2 and 95% O_2. In experiments in which the effects of alteration of Na^+ concentration were examined, a modified Locke solution was used (mM): NaCl 143.5, KCl 5.94, $CaCl_2$ 2.54, sodium phosphate buffer 1.0 (pH 7.3) and glucose 11; it was gassed with O_2. NaCl was replaced by an equivalent amount of sucrose; choline chloride was not used because it depolarizes the ganglion cells and their postganglionic fibres in this preparation (Kosterlitz *et al.*, 1968). The concentration of sucrose was 315 mM and taken to be isotonic. Unless otherwise stated, experiments were carried out at temperatures between 21° and 27° C and, in any one experiment, the temperature varied by less than 1° C.

Drugs

The drugs used were acetylcholine chloride, carbachol (carbamoyl choline) chloride, physostigmine sulphate, hexamethonium bromide, hyoscine hydrobromide and strophanthin G (ouabain). The stock solutions were made with modified Locke solution without phosphate buffer. The concentrations (μM) refer to the bases.

Results

Relation between after-hyperpolarization and acetylcholine-induced depolarization

In confirmation of our previous observations, it was found that an after-hyperpolarization did not occur in the postganglionic axons but occurred in the ganglion after depolarization with choline, acetylcholine (in the presence or absence of physostigmine, 60 μM) or carbachol. With increasing concentrations of acetylcholine, the amplitude of the after-hyperpolarization increased as did the amplitude and rate of depolarization (Figure 1a). The threshold concentration of acetylcholine, in the presence of physostigmine, required to produce an after-hyperpolarization was

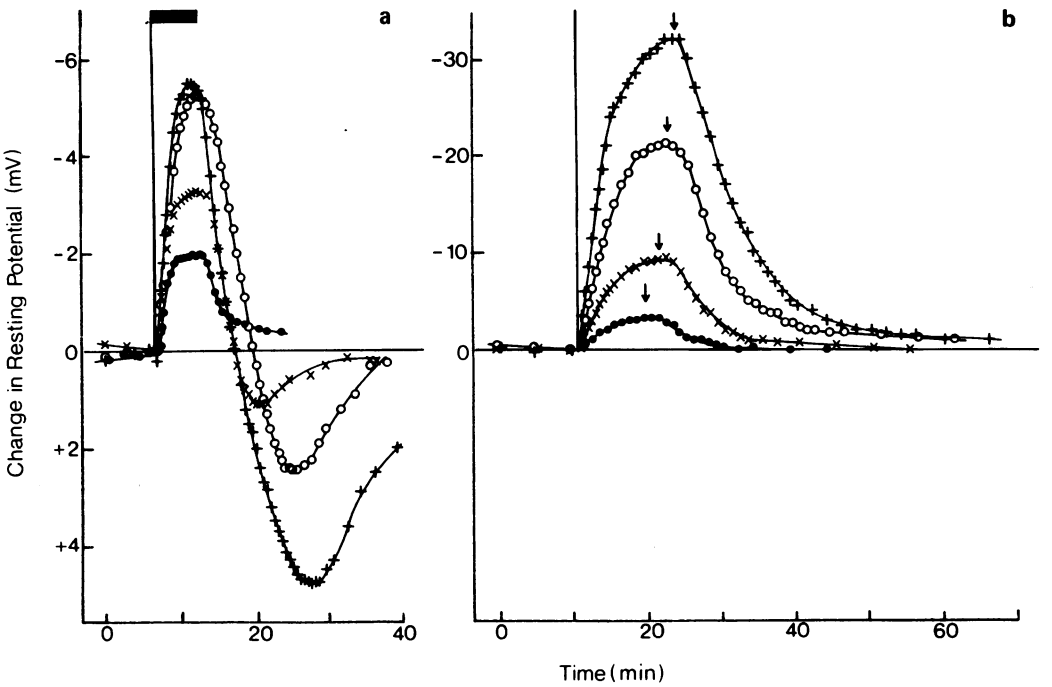

Fig. 1 Comparison of the effects of increasing concentrations of K^+ and acetylcholine (ACh) in the bathing fluid on ganglionic resting potential. (a) Increasing depolarization with subsequent after-hyperpolarization with increasing concentrations of ACh. Ordinate: depolarization upwards. Physostigmine (60 μM) throughout experiment. Solid bar, exposure to ACh. •, 5.5 μM; x, 16.5 μM; ○, 50 μM; +, 150 μM. (b) Increasing depolarization without subsequent after-hyperpolarization with increasing concentrations of K^+. Ordinates: note altered scale. Vertical line, exposure to K^+-rich solution until arrow. •, 12.5 mM K^+; x, 26.2 mM K^+; ○, 55 mM K^+; +, 115 mM K^+.

about 15 μM. It may be safely concluded that after-hyperpolarizations are not a consequence of large depolarizations of the cells because depolarizations induced with K^+ were never followed by after-hyperpolarizations (Figure 1b).

In agreement with our earlier observations (Kosterlitz *et al.*, 1968), it was found that, for a large after-hyperpolarization to be induced in any one ganglion, high concentrations (80-110 μM) of acetylcholine were required even in the presence of a maximally effective concentration of physostigmine (60 μM). The mean amplitude of after-hyperpolarization following the first depolarization in each of 35 experiments, was 4.87 ± 0.50 mV (mean ± s.e. mean); there was no consistent change in amplitude of the after-hyperpolarization following the first three depolarizations but the fourth was reduced by 30-50%. The mean amplitude of the corresponding first depolarization was 9.33 ± 0.47 mV (mean ± s.e. mean); the second depolarization was usually not less than 80% of the first, and the third and fourth depolarizations were both about 30% less than the first. There was a significant ($P < 0.01$) positive correlation between the amplitudes of the first depolarization and its associated after-hyperpolarization in 35 experiments, indicating some dependence of the after-hyperpolarization on depolarization magnitude.

When a high concentration of acetylcholine (80-110 μM) was used, the amplitude of the after-hyperpolarization was more closely related to the duration of exposure to acetylcholine than to the amplitude of the depolarization (Figure 2). It was found that with depolarizations lasting less than 1 min, there was no or only a small after-hyperpolarization (Fig. 2a & d), whereas, as the duration of depolarization was increased to a maximum of 12 min, the amplitude and duration of the after-hyperpolarization increased (Figure 2b & c). In confirmation of the result shown in Fig. 2c, it was found later in this experiment that an after-hyperpolarization evoked by an exposure to acetylcholine (110 μM) for 11 min had an amplitude of 3.6 mV (Figure 2e). This was 35% greater than the after-hyperpolarization following

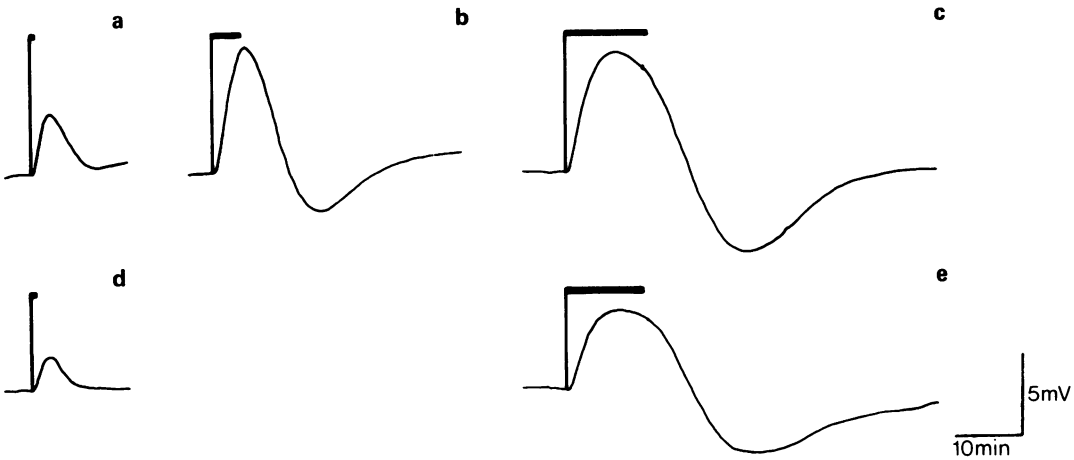

Fig. 2 Effect of time exposure to acetylcholine (ACh) on amplitude of after-hyperpolarization. Continuous recording, depolarization upwards. Solid bar, exposure to ACh (110 μM). (a) 40 s; (b) 4 min; (c) 11 min; (d) 40 s; (e) 11 min. Depolarizations began at the following times after setting up of preparation: (a) 1 h 30 min; (b) 2 h 30 min; (c) 3 h 24 min; (d) 4 h 37 min; (e) 5 h 30 min. Physostigmine (60 μM) throughout experiment.

an exposure to acetylcholine for 4 min (Fig. 2b), although the depolarization was only 67% of that attained during the shorter exposure, which had been carried out about 3 h earlier. In all other experiments, the duration of exposure to acetylcholine was held constant in any one experiment, the depolarization always being allowed to reach its maximum before washing out the acetylcholine. Commonly, an exposure time of 8-10 min was required.

Dependence upon external sodium ions

One possible explanation for these observations was that the after-hyperpolarization was related to, or a consequence of, the amount of Na^+ entering the cells during the preceding depolarization. Accordingly, attempts were made to prove this point directly by reducing the amount of Na^+ present in the extracellular fluid during the depolarization. In a preliminary experiment, the NaCl of Krebs solution was replaced with an osmotically equivalent amount of sucrose. Although the depolarization obtained was significantly reduced in amplitude, the striking change was a reduction in the rate of repolarization and onset of after-hyperpolarization as well as in amplitude of the after-hyperpolarization (Figure 3a & b). In order to reduce the Na^+ content of the bathing solution still further, a modified Locke

Table 1 Effect of low-sodium solutions on depolarization and subsequent after-hyperpolarization

	144.5 mM Na^+-Locke		*15 mM Na^+-Locke*	
Experiment	*Depolarization (mV)*	*After-hyperpolarization (mV)*	*Depolarization (mV)*	*After-hyperpolarization (mV)*
a	11.3	1.2	4.2	0.3
b	11.9	3.9	4.8	1.6
c	9.4	1.5	2.8	0.2
d	10.3	1.5	9.8	–†
e	3.6	2.3	2.6	–†
f*	5.9	4.5	5.8	3.3
g*	5.8	8.9	7.4	2.3

* In this experiment, ganglion exposed first to 15 mM Na^+-Locke.
† Incomplete repolarization.

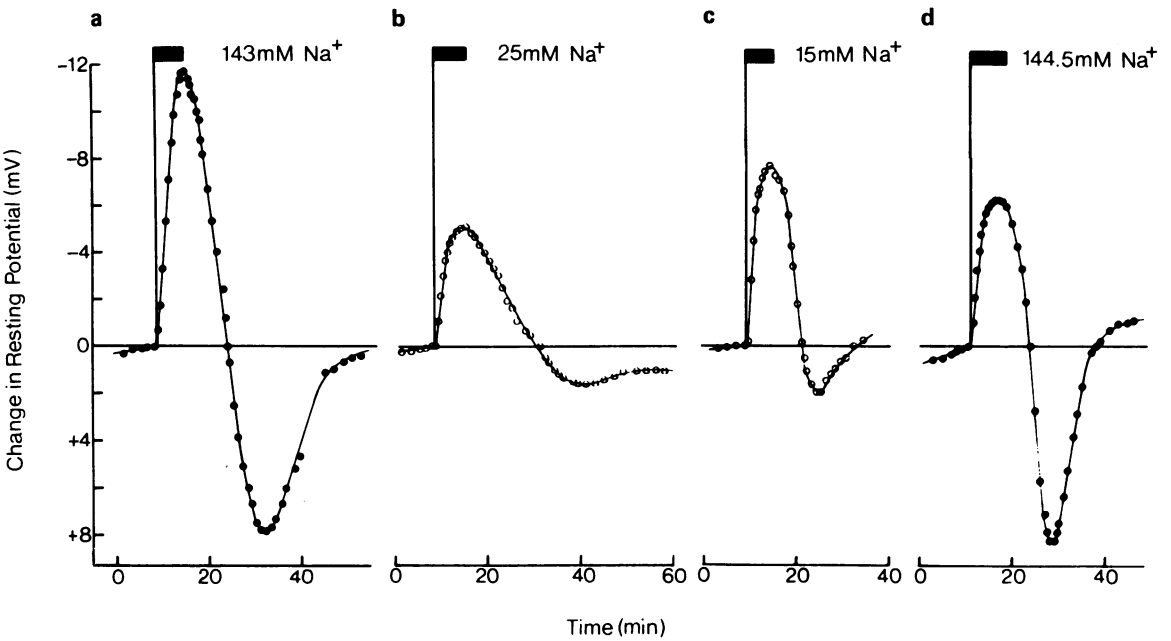

Fig. 3 Effect of reduction in external Na^+ concentration on the depolarization and after-hyperpolarization to acteylcholine (ACh) (two experiments). Ordinates: depolarization upwards. Solid bar, exposure to ACh (110 μM). Physostigmine (60 μM) throughout experiment. (a) In 143 mM Na^+-Krebs solution; (b) in 25 mM Na^+-Krebs solution, solution changed 35 min before depolarization by ACh; (c) in 15 mM Na^+-Locke solution, solution changed 44 min before depolarization by ACh; (d) in 144.5 mM Na^+-Locke solution, solution changed 55 min before depolarization by ACh.

solution was used. Figure 3c & d shows the results of one such experiment in which, in 15 mM Na^+-Locke solution, the after-hyperpolarization was only 25% of that obtained subsequently in 144.5 mM Na^+-Locke solution, although the depolarization was larger in the low-Na^+ solution. The amplitudes of the after-hyperpolarizations and corresponding depolarizations obtained in 15 mM Na^+-Locke solution are compared in Table 1 with those in 144.5 mM Na^+-Locke solution. For the interpretation of these results, it should be noted that, in 144.5 mM Na^+-Locke solution, the depolarization due to a second exposure to acetylcholine was reduced to a variable extent but not by more than about 50% of the first depolarization; in 143 mM Na^+-Krebs solution, the corresponding decrement, as stated earlier, was much less. The consistent finding was a reduction in the amplitude of the after-hyperpolarization in 15 mM Na^+-Locke solution.

Further support for the view that Na^+ were involved in the generation of the after-hyperpolarization might have been obtained from experiments in which Li^+ substituted for Na^+ in the bathing medium. Although Li^+ may substitute for Na^+ in axonal conduction in the internal carotid nerve (Kosterlitz, Lees & Wallis, 1970b), it would have been expected that the after-hyperpolarization would be abolished since Li^+ are not pumped out of cells (Ritchie & Straub, 1957; Keynes & Swan, 1959; Kerkut & Thomas, 1965). Unfortunately, it was not possible to test this hypothesis because, on changing to Li^+-containing Krebs solution, a large change in potential (−20 to −30 mV) occurred over 15-20 minutes. Under these conditions, no effect of acetylcholine was detected.

Effect of altering external potassium ion concentration

If the after-hyperpolarization were due to an increase in K^+ permeability, a reduction in external K^+ concentration would result in an increase in the amplitude of the after-hyperpolarization. In preliminary experiments, however, it had been found that, when K^+ were omitted from the bathing fluid for 20-30 min, the amplitude of the after-hyperpolarization was not increased while the rates of onset and offset of the potential

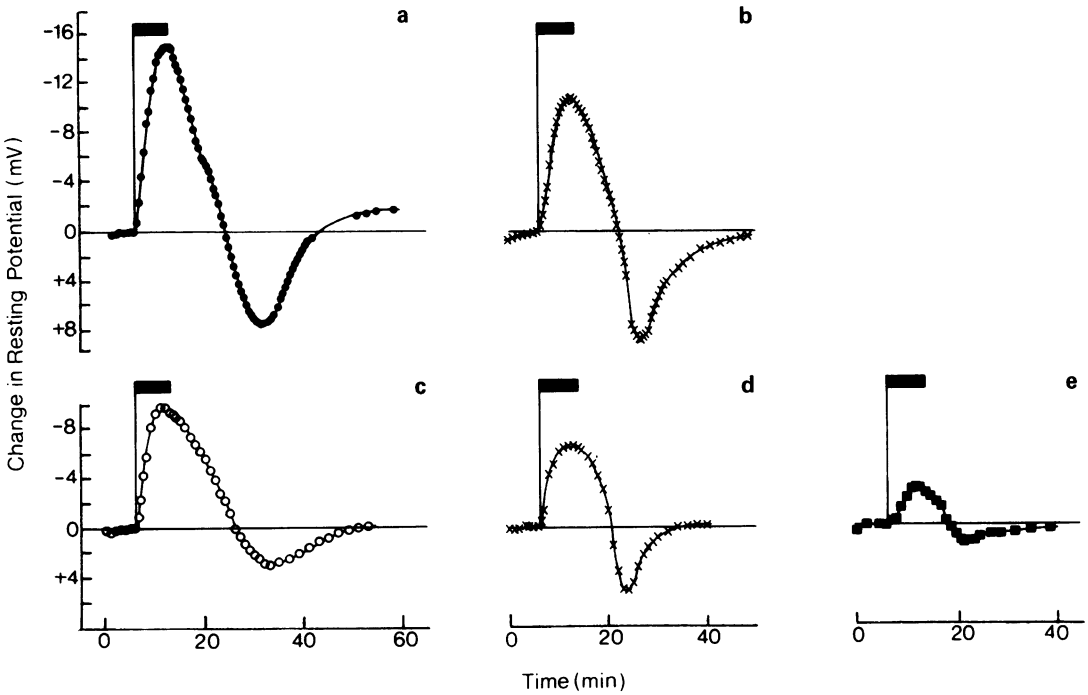

Fig. 4 Effects of varying concentration of K^+ in the bathing fluid $[K^+]_o$, on the amplitude and rate of development of the after-hyperpolarization (two experiments). In K^+-free solutions, the NaCl content was increased by an equivalent amount; in K^+-rich solutions, the KCl content was increased at the expense of NaCl. Ordinates: depolarization upwards. Solid bar, exposure to acetylcholine (ACh). (a) & (b) 110 μM; (c), (d) & (e) 150 μM. Physostigmine (60 μM) throughout experiments. Exposure of ganglia to changes in $[K^+]_o$ produced the following changes in resting potential with respect to control (6 mM K^+) values. (b) 12.5 mM K^+, −6.4 mV; (c) 0 mM K^+, +5.4 mV; (d) 12.5 mM K^+, −2.9 mV; (e) 26.2 mM K^+, −11.4 mV. (a) Ganglion exposed to 6 mM K^+-Krebs solution; (b) ganglion exposed to 12.5 mM K^+-solution, solution changed 32 min prior to depolarization by ACh; (c) ganglion exposed to K^+-free solution, commencing 29 min prior to depolarization by ACh; (d) ganglion exposed to 12.5 mM K^+-solution, solution changed 28 min before depolarization by ACh; (e) ganglion exposed to 26.2 mM K^+-solution, solution changed 20 min before depolarization by ACh.

Note in (b) and (d) increase in rates of onset and decline of after-hyperpolarization in 12.5 mM K^+-solution, as well as increased amplitude.

change were greatly reduced (Kosterlitz *et al.*, 1968). As the membrane potential approaches E_K, an increase in permeability to K^+ would be accompanied by a progressively smaller hyperpolarization. When 12.5 mM K^+-Krebs solution is used, it can be shown that the resting potential of ganglion cells is close to E_K (Kosterlitz *et al.*, 1968). Under these conditions, the amplitude of the after-hyperpolarization was unchanged or increased, while the rate of development was greatly increased (Figure 4). Even in 26.2 mM K^+-Krebs solution, a small but distinct after-hyperpolarization could still be observed following a much reduced acetylcholine-induced depolarization. These findings are in complete contrast to the effect of 12.5 mM K^+-Krebs solution on the positive after-potential evoked by orthodromic stimulation of the ganglion. The after-potential was much reduced or absent (Fig. 5), whereas in potassium-free Krebs solution the after-potential was very greatly increased.

The evidence presented so far is consistent with the active extrusion of Na^+ and a hyperpolarization generated as a consequence of this. However, it does not distinguish clearly between the potentials created by an electrogenic pump and by an electrically-neutral pump. A diffusion barrier around the cells might allow an electrically-neutral pump to generate the potential if the K^+ concentration within the barrier fell. This latter possibility was excluded by the following experiment: in K^+-free solution, acetylcholine caused a depolarization which declined slowly, to be followed by a small hyperpolarization. If at this

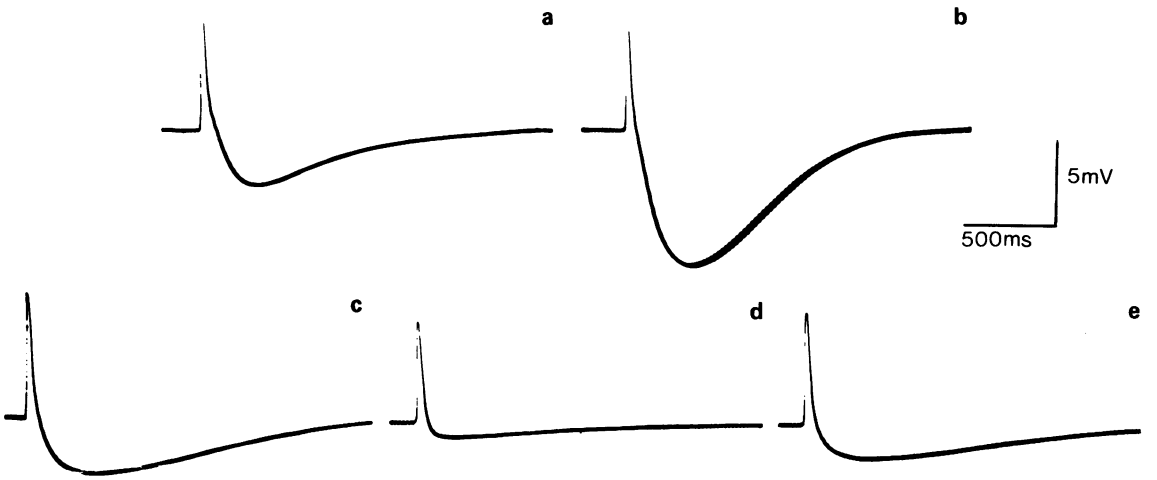

Fig. 5 Effect of varying concentration of K^+ in the bathing fluid $[K^+]_0$ on the amplitude of the after-potential and on the ganglionic resting potential. KCl replaced, or was replaced by, NaCl. $[K^+]_0$: (a) 6 mM; (b) K^+-free for 20 min; (c) 6 mM; (d) 12.5 mM for 20 min; (e) 6 mM.

time, the external K^+ concentration was increased to the original 6 mM, a large, rapid hyperpolarization immediately ensued (Kosterlitz *et al.*, 1970a). Although a neutral pump might have been stimulated by such a manoeuvre, the immediate change in the vicinity of the pump and thus inside the diffusion barrier must have been an increase in the K^+ concentration, which would have led to a depolarization of the cells. Therefore, the finding is consistent with the view (Rang & Ritchie, 1968) that hyperpolarization may be due to the activity of an electrogenic Na pump which requires K^+ extracellularly for its operation. Brown *et al.* (1972) recently obtained similar results in the rat superior cervical ganglion.

Cs^+ is a rather ineffective substitute for K^+ in stimulating sodium pumping in the unmyelinated axons of the rabbit vagus nerve (Range & Ritchie, 1968) and in the rat superior cervical ganglion (Brown *et al.*, 1972). In support of these observations, no, or only very small, after-hyperpolarizations were observed in two experiments in which the K^+ of Krebs solution were replaced by an equivalent amount (6 mM) of Cs^+. It was found, in one such experiment (Fig. 6), that in 6 mM Cs^+-Krebs solution, the after-hyperpolarization was reduced to 0.8 mV from a control value of 5.3 mV; when, however, 6 mM K^+-Krebs solution was reintroduced at the time of maximal hyperpolarization in the Cs^+ solution, a further, more rapid hyperpolarization ensued, with an amplitude of 5.3 mV. In both experiments, on changing from 6 mM K^+-Krebs solution to 6 mM Cs^+-Krebs solution, a small hyperpolarization (1 mV or less) occurred, suggesting perhaps that Cs^+ is not able to substitute completely for K^+ in maintaining the normal resting potential of the ganglion cells. On reintroducing K^+ as described above, the resting potential moved initially towards the expected value of the resting potential in 6 mM K^+-Krebs solution before the commencement of the hyperpolarization.

Effect of ouabain

The finding that the after-hyperpolarization is dependent on the simultaneous presence of both Na^+ and K^+, inside and outside the cell respectively, points to the operation of a process closely resembling many other Na-K exchange mechanisms, which have been shown to be driven by, or identical to, Na^+-K^+-activated ATPase. Many Na^+-K^+-activated ATPases are inhibited by cardiac glycosides in high concentrations. Although a reduction in the amplitude of the potential would not by itself be proof of a metabolically-dependent process (Kehoe & Ascher, 1970), it was of considerable importance to investigate the effect of a cardiac glycoside (ouabain) on the after-hyperpolarization of ganglion cells and it was tested in the following manner. The first depolarization by acetylcholine (110 μM) and recovery were carried out in Krebs solution; when the depolarization by acetylcholine during the second exposure was maximal, the Krebs solution containing acetylcholine (110 μM) was replaced by Krebs solution containing both acetylcholine (110 μM) and ouabain (10 μM) for 2

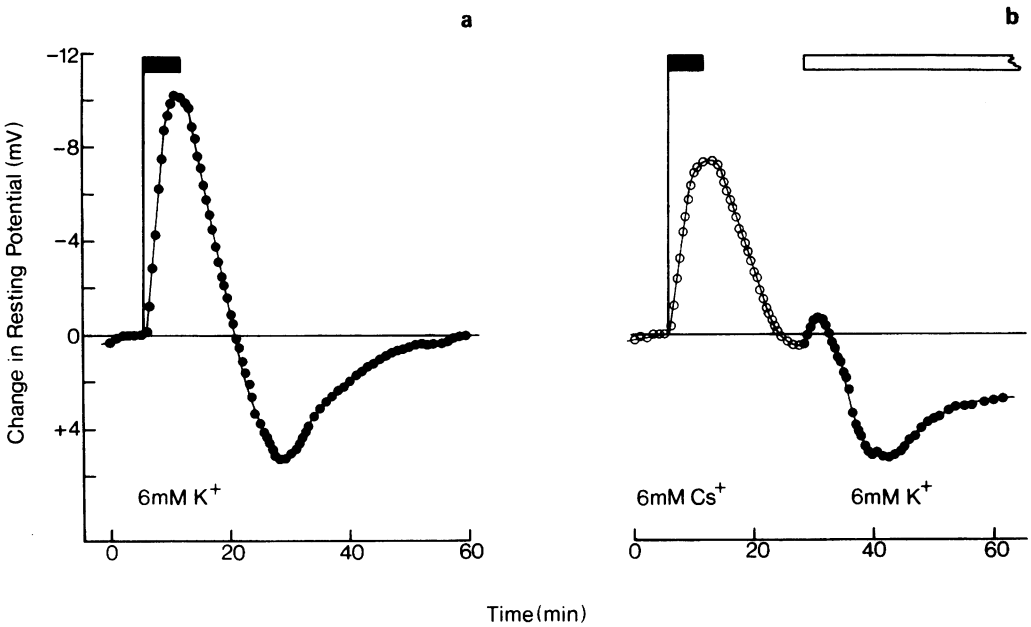

Fig. 6 Relative failure of Cs^+ to substitute for K^+ in activating the electrogenic Na pump and subsequent excitation of the pump by extracellular K^+. Ordinates: depolarization upwards. Solid bar, exposure to acetylcholine (ACh) (110 μM). Physostigmine (60 μM) throughout experiment. (a) •, in 6 mM K^+-Krebs solution; (b) ○, in 6 mM Cs^+-Krebs solution, solution changed 36 min before depolarization by ACh; •, changed to 6 mM K^+-Krebs solution from 6 mM Cs^+-Krebs solution at time indicated by the open bar. Note: on changing from 6 mM K^+-Krebs solution to 6 mM Cs^+-Krebs solution between (a) & (b) a small hyperpolarization (1 mV) occurred; on changing back to 6 mM K^+-Krebs solution, the ganglionic potential moved towards the expected value of the resting potential before the large after-hyperpolarization developed.

minutes. Recovery was then allowed to commence in Krebs solution containing ouabain (10 μM). As can be seen from Fig. 7, the after-hyperpolarization was prevented. In two other experiments, ouabain (10 μM) greatly reduced after-hyperpolarization, the remaining potential change in both cases, however, being accounted for by d.c. drift.

Metabolic dependence of the after-hyperpolarization

Since the metabolic dependence of Na-K exchange mechanisms is well known, it seemed important to show that the after-hyperpolarization was linked to the activity of an energy-consuming process. In confirmation of this, it was found in two of three experiments that the amplitude and rate of development of the after-hyperpolarization were reduced when the ganglion was bathed in a glucose-free Krebs solution (Figure 8). On returning to a glucose-containing solution, the rate of development of the after-hyperpolarization was increased although the amplitude of the potential did not recover.

As has already been stated, the temperature during any one experiment was kept constant, with the exception of a few special experiments. Contrary to expectation, it was found in 35 experiments that there was no significant increase in the mean amplitude of the after-hyperpolarization with increasing temperature over the range 20°-30°C. It was noticed, however, that, in five experiments at temperatures of 30°-33°C, the after-hyperpolarizations were smaller than usual or absent. In a single experiment in which the temperature was changed successively from 25.5°C, initially, to 21.5°C and, finally, to 27°C, the depolarizations and after-hyperpolarizations were: −8.8 mV, +5.0 mV; −7.5 mV, +6.5 mV; −4.3 mV, +1.5 mV respectively. Thus, although the depolarization declined throughout the experiment, the largest hyperpolarization was obtained at the lowest temperature. This tendency for the after-hyperpolarization to be smaller at higher temperatures may reflect reductions in membrane resistance such as occurs in other neuronal tissues. There was no correlation between the amplitude of the depolarization by acetylcholine and the

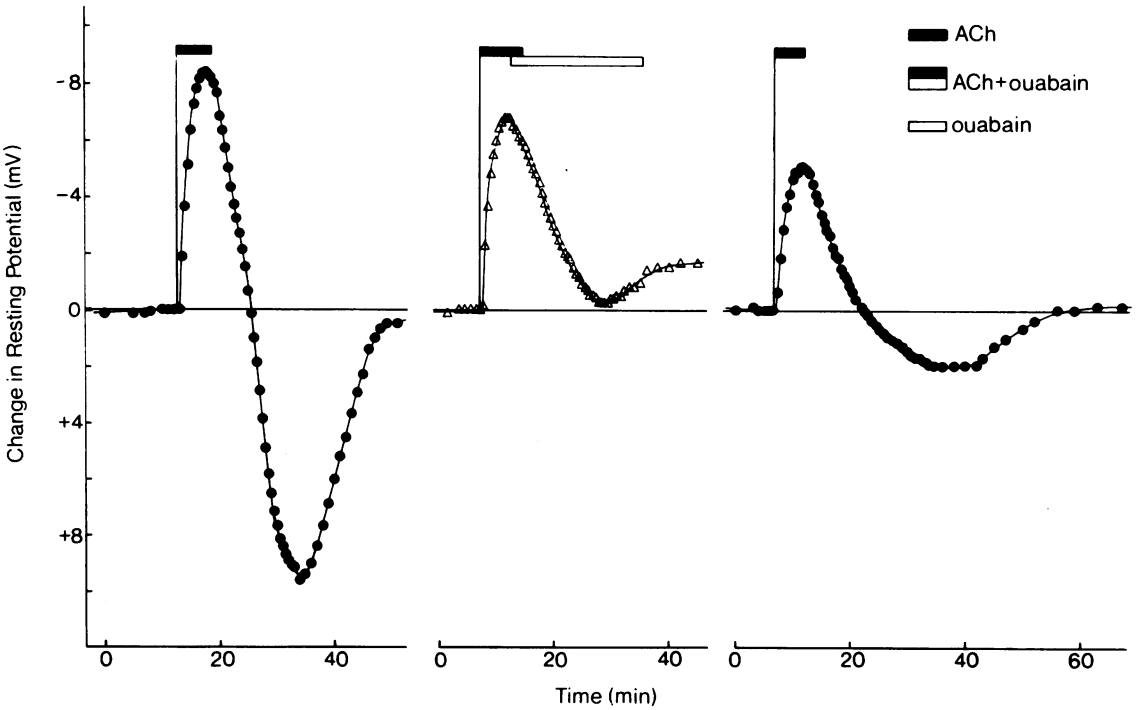

Fig. 7 Effect of ouabain (10 μM) on after-hyperpolarization. Ordinates: depolarization upwards. Physostigmine (60 μM) throughout experiment. For details of application of ouabain see text. Third depolarization by acetylcholine begun 57 min after discontinuing exposure to ouabain.

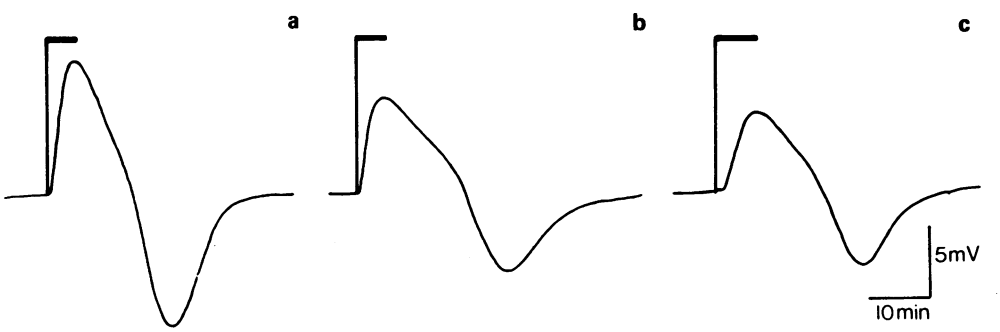

Fig. 8 Effect of glucose-free solution on amplitude and rate of development of after-hyperpolarization. Continuous recording, depolarization upwards. Solid bar, exposure to acetylcholine (ACh) (110 μM). Physostigmine (60 μM) throughout experiment. Temperature, 24° C. (a) Control; (b) ganglion exposed to glucose-free Krebs solution, solution changed 103 min before exposure to acetylcholine (ACh); (c) in glucose-containing Krebs solution, solution changed 42 min before depolarization by ACh.

temperature. No attempt was made to investigate the effect of a low temperature on the after-hyperpolarization.

Time course of the after-hyperpolarization

The decay of the after-hyperpolarization was found to be exponential after an initial slower rate of decay. Decay of the hyperpolarizations in Fig. 2b & c is plotted in Figure 9a. The time constants of the exponential portions of the two curves were similar, being 5.2 and 4.6 min respectively for the 11 and 4 min exposures; the corresponding rate constants were 0.19 and 0.22 min^{-1}. In keeping with these observations was the finding that the rate constants calculated from the exponential

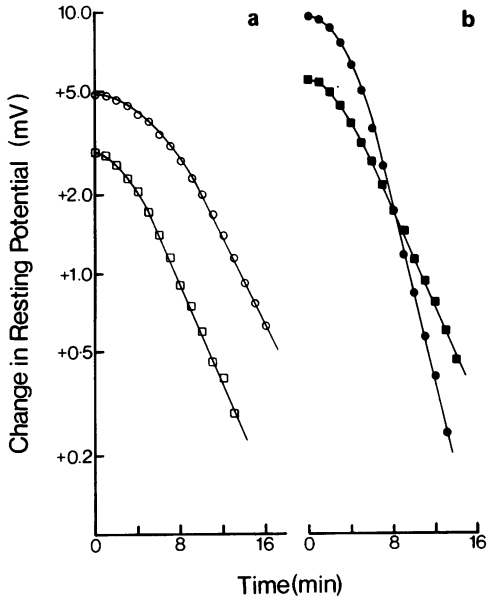

Fig. 9 Decay of after-hyperpolarization plotted on a semi-logarithmic scale against time. Ordinates: hyperpolarization upwards, logarithmic scale. (a) Decay of after-hyperpolarizations of response of Fig. 2(b), □, and of response of Fig. 2(c), ○; (b) decay of after-hyperpolarizations of response of Fig. 8(a), •, and of response of Fig. 8(b), ▪. On returning to Krebs solution containing glucose, the decay was similar to the control but the line has been omitted for clarity.

portions of the curves from results obtained in 15 mM Na^+-Locke and in 144.5 mM Na^+-Locke were similar, being 0.20 and 0.17 min^{-1}, respectively. For comparison, the decay of the after-hyperpolarization in glucose-free solution (Fig. 8b) is plotted in Figure 9b. The time constant (4.9 min) was considerably larger than the control value (2.6 minutes). This presumably reflects a reduced rate of pumping in the glucose-free medium.

The activity of the pump is also clearly influenced by the external K^+ concentration, as in the C fibres of the vagus nerve. The slowest rate constant of decay was seen in K^+-free solutions (0.07 and 0.11 min^{-1}); in 6 mM K^+-Krebs solution, the mean rate in four experiments was 0.23 min^{-1} and in 12.5 mM K^+, the rate was 0.18 and 0.23 min^{-1}

Effect of altering external calcium ion concentration

Since it is well known that Ca^{++} play an important part in determining membrane resistance, the effects of changes in Ca^{++} concentration of the bathing medium were studied on the depolarization and after-hyperpolarization. For brevity, a Krebs solution from which $CaCl_2$ was omitted will be referred to as Ca^{++}-free Krebs solution. There was a marked difference in the amplitudes of the after-hyperpolarization achieved in Ca^{++}-free Krebs solution compared to the controls; the after-hyperpolarization was greatly reduced or, more usually, abolished even after bathing the ganglion in Ca^{++}-free Krebs solution for only 30-40 min (Figure 10b). There was no evidence that the depolarization under these conditions was less than in 2.54 mM Ca^{++}-Krebs solution. In addition, it was found that increasing the Ca^{++} concentration to 3.8 mM further increased the amplitude of the after-hyperpolarization (Figure 10f). It was possible that during depolarization in Ca^{++}-Krebs solution, Ca^{++} entered the cells and their subsequent extrusion was the basis of the after-hyperpolarizations. Such a possibility, however, was ruled out by the results of experiments in which the depolarization and recovery were carried out in Ca^{++}-free Krebs solution until the maximal repolarization was reached. At this point, 2.54 mM Ca^{++}-Krebs solution replaced the Ca^{++}-free Krebs solution and a further repolarization or, more usually, a hyperpolarization occurred.

Comparison with responses to orthodromic stimulation of ganglion

When ganglionic potentials are recorded by extra-cellular techniques in the presence of high concentrations of hexamethonium or (+)-tubocurarine, it is difficult to refute that action potentials are being generated in some cells in response to orthodromic stimulation in view of the fact that some synapses are not easily blocked by these drugs (Kosterlitz & Wallis, 1966; Kosterlitz *et al.*, 1970b). Kosterlitz *et al.* (1968) considered it impossible to exclude that both mechanisms mentioned above (i.e. positive after-potential and slow i.p.s.p.) were contributing to some (possibly variable) extent to the positive ganglionic potential following any orthodromic stimulation of the ganglion. They, therefore, referred to that potential occurring in the presence or absence of a nicotinic receptor blocking agent as the P wave. It is in this sense that the term P wave is used below.

In confirmation of previous observations, it was found that, in the presence of hexamethonium (276 μM), the amplitude of the P wave was greater after a short train (1 s) of supramaximal stimuli applied to the preganglionic nerve at 10 Hz than after a single stimulus. (Physostigmine was not present in the bathing solutions.) When K^+-free solution now bathed the ganglion, the amplitude of the P wave increased. Once the P wave appeared

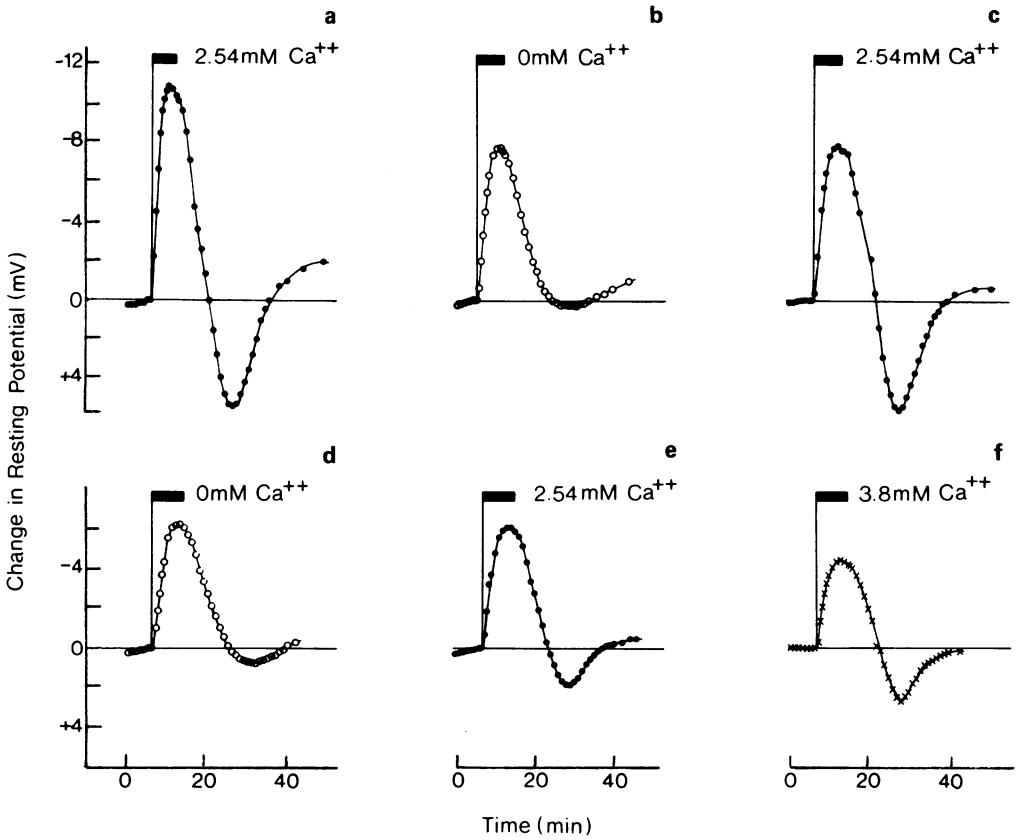

Fig. 10 Effect of varying the Ca^{++} concentration of the bathing fluid on the amplitude of the after-hyperpolarization (two experiments). Ordinates: depolarization upwards. Solid bar, exposure to acetylcholine (ACh) (110 μM). Physostigmine (60 μM) throughout experiments. (a) In 2.54 mM Ca^{++}-solution; (b) Ca^{++} omitted from bathing fluid (0 mM Ca^{++}), solution changed 42 min before exposure to ACh; (c) Ca^{++} omitted from bathing fluid 84 min before exposure to ACh; (d) in 2.54 mM Ca^{++}-solution for 25 min before exposure to ACh; (e) in 3.8 mM Ca^{++}-solution, solution changed 29 min before exposure to ACh.

to be maximally potentiated, the ganglion was exposed to ouabain (10 μM) in the continued absence of K^+. This concentration of ouabain was chosen because it had blocked the after-hyperpolarization within 10 min; the absence of K^+ would be expected to increase the effectiveness of ouabain. There was no indication of any reduction in the amplitude of the P wave even after 20 min exposure to ouabain (Figure 11). When ouabain was used in concentrations greater than this, there was a reduction in the size of the preceding compound synaptic potential (N wave) as well as a gradual decline in the amplitude of the P wave. The finding that the P wave was not readily inhibited is in accordance with the results of Kobayashi & Libet (1968) and Libet & Kobayashi (1969) for this ganglion.

Contribution of a 'chloride shunt'

The after-hyperpolarization could be varied greatly in amplitude without changing the external Cl^- concentration; it seems most unlikely, therefore, that the after-hyperpolarization could be due to the inward movement of Cl^-. Cl^- might, however, influence the amplitude of the potential in quite another manner. Outward movement of Cl^- together with Na^+ has been shown to prevent the full amplitude of the post-tetanic hyperpolarization from being developed in non-myelinated fibres of the cervical vagus nerve of the rabbit (Rang & Ritchie, 1968). In the superior cervical ganglion, however, it was not possible to determine to what extent the after-hyperpolarization is diminished as a result of the Cl^- shunt

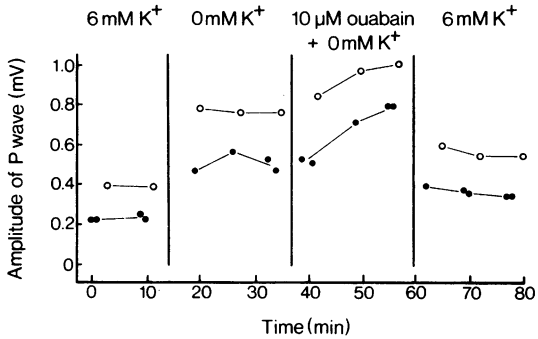

Fig. 11 Lack of effect of ouabain (10 μM) on amplitude of P wave of ganglion. •, single supramaximal stimulus; ○, 11 supramaximal stimuli at 10 Hz. 6 mM K^+, control obtained in Krebs solution; 0 mM K^+, in K^+-free solution. Hexamethonium (276 μM) present throughout experiment.

because, on replacing Cl^- by SO_4^{--} or isethionate, there was a change in potential of the ganglion. This was assumed to be a depolarization of the ganglion cells since acetylcholine could induce only a very small depolarization in comparison with the control. In one of two experiments in which the initial depolarization on changing to Cl^--free solutions was small, there was an increase in amplitude of the after-hyperpolarization (3.0 mV to 7.0 mV); in the other, there was no increase. Drug-induced after-hyperpolarizations following exposure to acetylcholine did not occur in the postganglionic fibres of the internal carotid nerve, even when Cl^- was replaced by SO_4^{--} or isethionate.

Discussion

It is clear that various substances which depolarize ganglion cells also produce a hyperpolarization of the cell membrane on their removal; this after-hyperpolarization is apparently mediated by nicotinic receptors. That muscarinic receptors are not involved is supported by our own observations that after-hyperpolarizations are not seen with acetyl-β-methyl-choline and by Watson's findings (1972) that neither pilocarpine nor McN-A-343 (4-*m*-chlorophenylcarbamoyloxy-2-butynyl trimethylammonium chloride) induces after-hyperpolarizations. Furthermore, hyoscine (0.23 μM) does not reduce the amplitude of the after-hyperpolarization, a finding which is in good agreement with the report of Brown *et al.* (1972) that the after-hyperpolarizations of rat ganglia are unaffected by atropine or hyoscine. Thus, there is no evidence from these experiments for the muscarinic H-receptors, mediating hyperpolarization, proposed by Volle (1966) and Volle & Pappano (1968).

However, activation of nictotinic receptors is not obligatory for the after-hyperpolarization. Although depolarization by a solution with a raised K^+ concentration does not produce an after-hyperpolarization on its removal, activation of ganglionic tryptamine receptors by 5-hydroxytryptamine produces pronounced after-hyperpolarizations after removal of the drug (Wallis & Woodward, unpublished).

Since the observations in solutions in which the K^+ concentration was changed are inconsistent with a hyperpolarization due to an increased K^+ conductance, passive movement of K^+ is not responsible for the generation of the after-hyperpolarization. Likewise, the experimental evidence is also against inward movement of Cl^- being involved in the production of the after-hyperpolarization. Thus, the remaining likely explanation of the after-hyperpolarization is that it is due to the activity of a metabolically dependent process extruding Na^+. Evidence in favour of this view is provided by the findings that (a) the amplitude of the after-hyperpolarization is diminished when the external Na^+ concentration is reduced before and during the depolarization by acetylcholine and when the time allowed for the depolarization is reduced; (b) the after-hyperpolarization is still present when the membrane potential is at or close to E_K; (c) the amplitude of the potential is not enhanced by increasing $[K^+]_i/[K^+]_o$ and is frequently much reduced; (d) the rate of change of the potential is positively correlated with increasing $[K^+]_o$, within the range 0-12.5 mM K^+; (e) the after-hyperpolarization is readily prevented or reduced by ouabain; and (f) the rates of onset and decay are reduced in glucose-free solutions. Furthermore, from the evidence of Pascoe (1956) and Brown *et al.* (1972), the after-hyperpolarization is depressed at low temperatures (below 20° C).

The amplitude of the after-hyperpolarization due to extrusion of Na^+ would be expected to be proportional to the increment in internal Na^+ concentration produced by acetylcholine, providing the coupling ratio of the pump and the membrane conductance remain constant. Rang & Ritchie (1968) have presented a quantitative treatment of these relationships for the post-tetanic hyperpolarization of vagal C fibres, but this treatment is inappropriate for our more limited data. They found that the decay of the post-tetanic hyperpolarization of unmyelinated vagal fibres was exponential with the exception of an initial component, which decayed faster. If the

tetanus were evoked in a K^+-free solution, they found that the addition of K^+ evoked a hyperpolarization; the decay of this K^+-evoked hyperpolarization was quite strictly exponential. Rang & Ritchie considered that the rate constant of decay reflected the rate of Na^+ extrusion, assuming the membrane permeability and the coupling ratio of the pump remained unchanged, and that the area under the hyperpolarization-time curve would be proportional to the total amount of Na^+ extruded (see also Brown *et al.*, 1972). Our results show that, as the concentration of acetylcholine was raised from threshold levels, after-hyperpolarization amplitude increased, as did depolarization amplitude. The latter, however, reached a maximum (Fig. 2) with a particular duration of exposure to a high concentration of acetylcholine, whereas, with longer exposures, after-hyperpolarization amplitude was further increased. The area of the after-hyperpolarization also increased, but the time constant of its exponential decay was unchanged (Fig. 9a). These findings would be expected if higher concentrations or longer exposures to a high concentration of acetylcholine produced greater increments in internal Na^+ concentration and the rate of Na^+ extrusion increased with increasing Na loads. Brown *et al.* (1972) reported that, in rat ganglia, the rate constant was not consistently affected by variation in carbachol concentrations from 110-580 μM. The dependence of the after-hyperpolarization on increases in internal Na^+ concentration is supported by our results with Na^+-deficient solutions, where the after-hyperpolarization was very much reduced, even though in some experiments the depolarization was apparently unaffected. The rate constants in 15 mM Na^+-Locke and in 144.5 mM Na^+-Locke were, however, very similar.

The results were entirely consistent with the potential being due to active extrusion of Na^+ by a Na pump, but further evidence was required to decide between a neutral pump operating within a diffusion barrier and an electrogenic pump. The experiment in which K^+ was added to the external medium, following an acetylcholine depolarization in a K^+-free solution, provided crucial evidence on this point. It is difficult to see how an electrically neutral pump could generate a hyperpolarization under these circumstances. Thus, the basis of the after-hyperpolarization would seem to be an electrogenic Na pump with a requirement for extra-cellular K^+. The pump presumably transports some K^+ inwards but there is no one-to-one exchange with outwardly transported Na^+; the coupling ratio is not unity. A very similar electrogenic pump is thought to be present in the membrane of the unmyelinated axons of the rabbit vagus nerve (Rang & Ritchie, 1968) and is partly responsible for the post-tetanic hyperpolarization observed. Why then was there no evidence for it in the unmyelinated axons of the rabbit internal carotid nerve in our experiments? Since drug-induced depolarizations of the axonal membrane are very small in amplitude (Kosterlitz *et al.*, 1968), the inward flux of Na^+ is presumably, not large enough to induce detectable electrogenic pumping of Na^+ even when factors short-circuiting the membrane, such as Cl^-, are removed.

Although the coupling ratio of the pump is not unity, it is not possible to say whether the ratio is fixed or variable with intracellular or extracellular ionic concentrations. It is tempting to suppose that there is a fixed coupling-ratio imposed on the pump by the stoichiometry of the system. In this case, electrogenic pumping of Na^+ might be a normal physiological feature of the ganglion cell contributing a fraction of the resting membrane potential as it does in certain invertebrate neurones (Thomas, 1972); this is of interest because the data of Kosterlitz *et al.* (1968) on the relationship between resting potential and external K^+ concentration indicate that Na^+ permeability may be an important determinant of resting potential.

The absolute dependence of Na^+ extrusion on external K^+ has not been demonstrated in these experiments, since large after-hyperpolarizations were sometimes seen in K^+-free solutions; in addition, the area under the after-hyperpolarization-time curve is greatest for responses in K^+-free solutions. This does not necessarily mean that the amount of Na extruded was greater in these solutions, for the rate of onset and the time constant of decay were noticeably slower in K^+-deficient solutions. It is unlikely that a superfusion medium free of K^+ would wash all K^+ from the extracellular space in the tissue but it is probable that efflux of K^+ from the cells, particularly during the prolonged depolarization, provides sufficient K^+ for Na pumping. However, it is insufficient for the normal rate of pumping observed in 6 mM K^+-Krebs solution. The areas of the after-hyperpolarizations declined as the K^+ concentration increased and, since this does not appear to reflect any decrease in Na pumping, it reflects either a change in resting membrane conductance or, possibly, a change in the coupling ratio of the pump. A conductance increase would partially short-circuit the pump and diminish both the amplitude and the area of the after-hyperpolarization. Although K^+ may have some short-circuiting effect, there was no evidence of an anion shunt due to Cl^-, unlike the situation in rabbit vagal C fibres (Rang & Ritchie, 1968). These authors did not report any effect of Ca^{++}

on Na pumping, but in ganglion cells the effects are dramatic. The great reduction or abolition of the after-hyperpolarization in the absence of Ca^{++} may again reflect changes in resting membrane conductance. It is less likely that the activity of the pump was directly affected by Ca^{++} concentration, e.g. through the activity of a Ca^{++}-dependent Na^{+}-K^{+} ATPase, since the time constants of the exponential portion of the decay of the after-hyperpolarization in 2.54 mM and 3.8 mM Ca^{++}-Krebs solution were very similar; in K^{+}-free Krebs solution, the time constant was more difficult to measure accurately but appeared to be unaltered. In support of an indirect effect of Ca^{++}, Brown *et al.* (1972) found that the after-hyperpolarization, much reduced in Ca^{++}-free Krebs solution, was restored on adding Mg^{++}; they attributed this to a decrease in membrane conductance in the presence of Mg^{++}.

After-hyperpolarizations can be evoked *in vivo*, for Gebber & Volle (1966) observed them following the depolarization induced by injecting tetramethylammonium into the cat superior cervical ganglion; further, this after-hyperpolarization was prevented by prior administration of ouabain. Hancock & Volle (1969) obtained after-hyperpolarizations with dimethylphenylpiperazinium in the same preparation. These results are important because they demonstrate that the hyperpolarization engendered by electrogenic Na pumping is still present at 37° C and is not short-circuited by increased membrane conductance. However, our experiments appeared to indicate that, above 30° C, increased short-circuiting of the potential might diminish its amplitude, even though the rate of Na extrusion is presumably increased.

The slow i.p.s.p. of amphibian ganglion cells has been tentatively ascribed to the stimulation of an electrogenic Na pump (Nishi & Koketsu, 1968), in part because it was sensitive to cooling and to ouabain. Libet & Kobayashi (1969) reported that the slow i.p.s.p. of rabbit ganglion cells was not selectively depressed by ouabain and concluded that a ouabain-sensitive electrogenic Na pump was not involved. The potential, however, shows a strong metabolic dependence and recent work has suggested that it is mediated by dopamine and cyclic AMP (Libet & Tosaka, 1970; McAfee & Greengard, 1972). In our experiments, the P wave, which in the presence of a high concentration of hexamethonium is presumably mainly a slow i.p.s.p., was not selectively depressed by ouabain, in agreement with the findings of Libet and his co-workers. However, not only was it potentiated by repetitive orthodromic stimulation in the expected manner, it was also potentiated by K^{+}-free solutions. The magnitude of this increase (about two-fold) was less than that of the post-spike positivity in the unblocked ganglion, even though the magnitude of the latter potentials was far greater. Thus, the post-spike positivity was 2.7-2.8 mV after a single stimulus and 4.2-4.3 mV after a 10 Hz train for 1 s; these values were increased three-fold in K^{+}-free solutions. It does not follow that the slow i.p.s.p. is due to a phase of increased K^{+} permeability; indeed, no detectable increase in conductance occurs (Libet & Kobayashi, 1969). The increased amplitude in K^{+}-free solution might be explained if the slow i.p.s.p. is generated by a fall in Na conductance induced by transmitter action, as has been proposed recently for the slow i.p.s.p. in amphibian ganglion cells (Weight & Padjen, 1972).

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