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Origin of the after-hyperpolarization that follows removal of depolarizing agents from the isolated superior cervical ganglion of the rat

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Summary

1. Potential changes in isolated rat superior cervical ganglia following addition and removal of depolarizing agents were recorded using a moving-fluid extracellular electrode system.

2. Ganglionic negativity produced by carbachol was followed by a pronounced ganglionic positivity on washing. This after-positivity was attributed to hyperpolarization of the ganglion cells since it was unaffected by crushing the postganglionic trunk.

3. The after-hyperpolarization was selectively depressed by (a) cooling (Q_{10} 2·3), (b) metabolic inhibitors (cyanide, azide, 2,4-dinitrophenol), (c) reducing $[K^+]_o$ or substituting Cs^+ for K^+, (d) ouabain, and (e) substituting Li^+ for Na^+. This suggested a close dependence on active Na^+ transport.

4. When K^+ was restored to K^+-free solution, or the preparation was warmed rapidly, or when metabolic inhibitors were washed away, the hyperpolarization was rapidly regenerated. The effect of restoring K^+ indicated that the hyperpolarization was generated directly by the Na^+ pump.

5. The hyperpolarization was not altered by replacing Cl^- with isethionate, indicating that the voltage change produced by the Na^+ current was not modified by passive Cl^- movements.

6. Hexamethonium added to the washout fluid augmented the after-hyperpolarization, suggesting that there was a high (cationic) leak current due to continued receptor-activation on washing with normal Krebs solution.

7. The hyperpolarization was reduced by omission of Ca^{2+} and restored by addition of Mg^{2+}. This was considered to result from changes in passive membrane permeability.

8. The time-course of post-carbachol hyperpolarization accorded with a Na^+ extrusion process whose rate was directly proportional to $[Na^+]_i$ with a rate constant of $0{\cdot}38 \pm 0{\cdot}02$ min^{-1} at 23–27° C.

9. With increasing concentrations of carbachol, the amplitude of the hyperpolarization increased in proportion to the preceding depolarization, but the rate constant of the hyperpolarization was unchanged.

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10. The after-hyperpolarization was reduced in proportion to the depolarization by hexamethonium, but was not affected by atropine, hyoscine or tetrodotoxin.

11. A hyperpolarization also followed depolarization by acetylcholine or by EDTA in Ca^{2+}-free solution.

12. Nicotine-depolarization was not followed by a hyperpolarization unless hexamethonium was added to the washout fluid. This was attributed to prolonged receptor-stimulation by nicotine.

13. It was concluded that the after-hyperpolarization was due to the electrogenic extrusion of Na^{+} accumulated in the ganglion cells during the preceding depolarization, with no involvement of specific muscarinic receptors. The relationship of this process to post-tetanic hyperpolarization and to other forms of drug-induced ganglionic hyperpolarization is discussed.

Introduction

The extrusion of Na^{+} from neurones may be incompletely coupled with K^{+} uptake, and so may produce membrane hyperpolarization. Such ' electrogenic' Na^{+} extrusion has been shown to occur following injection of Na^{+} into invertebrate neurones (Kerkut & Thomas, 1965 ; Nakajima & Takahashi, 1966 ; Thomas, 1969) and appears to be responsible for post-tetanic hyperpolarization in both invertebrate neurones (Nakajima & Takahashi, 1966 ; Baylor & Nicholls, 1969) and vertebrate nerve fibres (Rang & Ritchie, 1968 ; Kuno, Miyahara & Weakly, 1970).

In isolated sympathetic ganglia a pronounced hyperpolarization accompanies recovery from acetylcholine or carbachol-induced depolarization (Pascoe, 1956 ; Brown, 1966a & b ; Kosterlitz, Lees & Wallis, 1968). The latter authors suggested that this after-hyperpolarization might also be due to electrogenic Na^{+} extrusion, because it was relatively insensitive to increasing external K^{+} concentrations.

In the present experiments this possibility has been examined more extensively, using isolated rat superior cervical ganglia with carbachol as the usual depolarizing agent. In particular the properties of post-carbachol hyperpolarization have been compared with those of post-tetanic hyperpolarization in mammalian non-myelinated axons described by Rang & Ritchie (1968).

Some of the observations described in this paper have been briefly reported elsewhere (Brown, Brownstein & Scholfield, 1969 ; Brown & Scholfield, 1970). Concurrently, Kosterlitz and his colleagues have been continuing their investigations on post-acetylcholine hyperpolarization in isolated rabbit ganglia, and have arrived at similar conclusions concerning the mechanism of this type of ganglionic hyperpolarization (Kosterlitz, Lees & Wallis, 1970 ; Lees & Wallis, personal communication).

Methods

Rats of approximately 200 to 250 g weight (either sex) were anaesthetized with urethane (1·5 g/kg intraperitoneally). The superior cervical ganglion, with pre- and postganglionic nerve trunks, was removed and the connective tissue sheath stripped from the ganglion and from most of the nerve trunks.

Ganglionic surface potential changes were recorded using the 'moving-fluid' electrode technique, as originally applied to ganglia by Pascoe (1956) with modifications described by Brown (1966a). The ganglion was mounted vertically with the postganglionic nerve uppermost in a bath containing 50 ml Krebs solution. The potential difference between the ganglion surface (recorded through the fluid meniscus) and the thread attached near to the cut end of the postganglionic nerve trunk was recorded using silver/silver chloride electrodes, one placed in the bath fluid and the other on the thread. The electrodes were connected through a DC amplifier to an oscilloscope or potentiometric X-Y recorder. Recordings were made by lowering the fluid meniscus down the length of the preparation, the movement being converted to a horizontal deflexion of the oscilloscope beam or the X-axis of the recorder (Burns & Paton, 1951): the resultant potential record formed a profile of the ganglionic surface potential with respect to the postganglionic trunk (see Fig. 1). Changes in the profile at various times following addition and removal of depolarizing agents were measured at the position of maximum difference from the control profile, to give time-plots of potential changes (Fig. 2). The principal advantage of this technique, apart from its simplicity, is that of providing a stable inter-electrode potential over the long periods required for measuring the drug-induced potential changes: the meniscus sweeps were rapid (<5 s) and the electrodes were shortened through the bath fluid between sweeps, during which period amplifier drift and slow fluctuations of inter-electrode potential were compensated. Also, since both electrodes were in contact with the same bath fluid, the effect of electrode-fluid junctional potential changes (a problem with the sucrose-gap method) was minimized. A disadvantage of the method, that of localization of potential changes, is discussed below.

The composition of the Krebs solution was (mM): NaCl, 118; KCl, 4·8; $CaCl_2$, 2·52; $NaHCO_3$, 25; KH_2PO_4, 1·18; $MgSO_4$, 1·19; glucose 11. Modified solutions were prepared as follows: *lithium solution*—NaCl replaced by LiCl (118 mM Li^+, 25 mM Na^+); *potassium-free solution*—KCl replaced by NaCl and KH_2PO_4 by NaH_2PO_4 (149 mM Na^+, 0 mM K^+); *caesium or rubidium solution*—KCl replaced by CsCl or RbCl, and KH_2PO_4 by NaH_2PO_4 (144 mM Na^+, 4·7 mM Cs^+ or Rb^+); *calcium-free solution*—$CaCl_2$ omitted; *isethionate solution*—NaCl replaced by Na^+ isethionate and KCl by 2·34 mM K_2SO_4 (118 mM isethionate, 2·52 mM Cl^-); *potassium solution*—addition of KCl to 20 mM K^+ or substitution of K_2SO_4 (59 mM) for NaCl to give 124 mM K^+, 25 mM Na^+. In some Li^+ solutions all of the Na^+ was replaced by Li^+, using Li_2CO_3 instead of $NaHCO_3$. To test lanthanum, $NaHCO_3$ and KH_2PO_4 were replaced by 5 mM trishydroxymethylaminomethane (Tris-buffer) and NaCl increased to 140 mM. Solutions were pre-equilibrated with 95% oxygen/5% carbon dioxide, to give a measured pH of 7·4; they were not usually bubbled during recording to prevent mechanical disturbance. Experiments were carried out normally at room temperature (22 to 27° C $\pm$ 1° C per day): hyperpolarization is temperature-sensitive (Pascoe, 1956; see also Fig. 6) but these daily fluctuations were too small to affect the observations seriously since the experiments were internally controlled. Temperatures outside this range were obtained by circulating warm or cold water through the outer jacket of the bath, the bath temperature being monitored with a thermistor.

The depolarizing agent was usually added directly to the bath fluid to give the final bath concentration indicated. Carbachol was added as the chloride salt and

nicotine as the free base. Other agents were added to the bath from a previously-prepared solution in a reservoir maintained at the same temperature as the recording bath.

Results

Interpretation of potentials

With the moving-fluid electrode technique, the entire length of the preparation, that is, the ganglion plus its pre- and postganglionic nerve trunks, is exposed to the same bath fluid. Consequently a change in the potential difference recorded between the ganglion and the postganglionic nerve trunk when the composition of the bath fluid is altered does not necessarily indicate a change in the ganglion cell membrane potential. Thus, if carbachol were to depolarize both ganglion and post-ganglionic trunk (*cf.* Armett & Richie, 1961 ; Kosterlitz *et al.*, 1968), an apparent ganglionic after-positivity on washing might reflect ganglion cell hyperpolarization, or might simply mean that the ganglion repolarized before the postganglionic trunk.

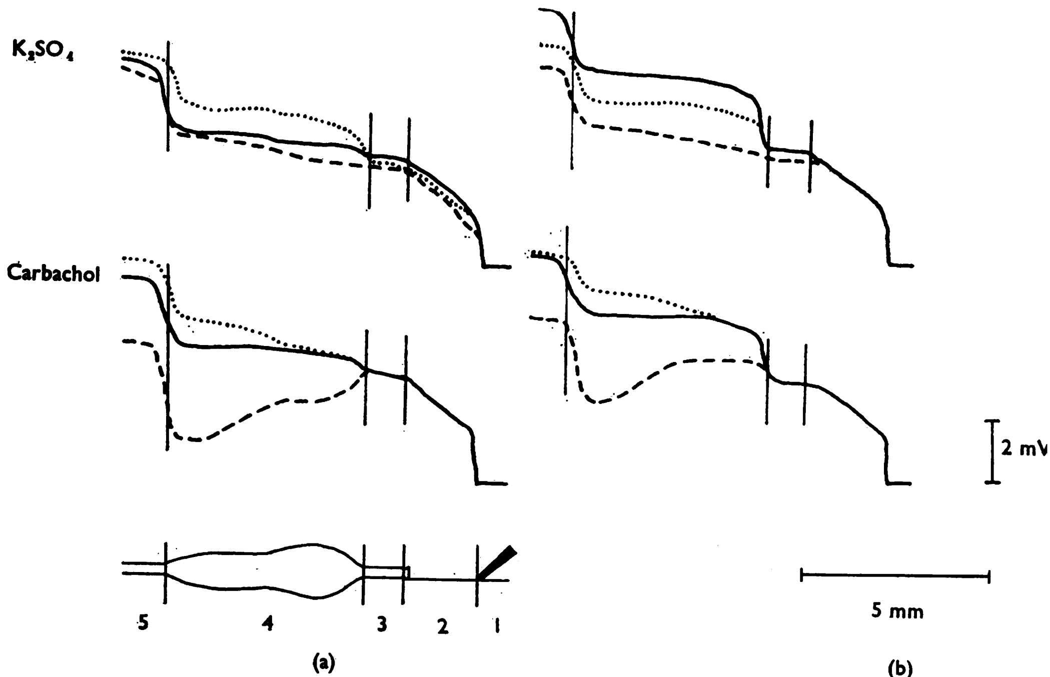


FIG. 1. Profile of surface potentials from an isolated rat superior cervical ganglion obtained on moving the fluid meniscus along the preparation, starting from the reference electrode (1), and then along the supporting thread (2), postganglionic trunk (3), ganglion (4), and preganglionic trunk (5) (ganglionic positivity upwards). Records were made (*a*) some hours after dissecting the preparation and then (*b*) within 30 min of crushing the postganglionic trunk: this procedure increased the demarcation potential between the ganglion surface (4) and postganglionic trunk (3) —— control sweep ; – – – 2 min after adding depolarizing agent to the bath (59 mM K_2SO_4 in upper records, 110 μM carbachol in lower records) ; · · · · ·, 1 min after washing out K_2SO_4 and 7 min after washing out carbachol. (In this experiment there was a potential drop along the supporting thread, because it had a high resistance, about 200 KΩ: in other experiments a thicker, lower-resistance thread was used. The electrode resistance was about 5–10 KΩ.) Key: —— control, – – – drug, · · · · · wash.

To check this the potential changes produced by carbachol were compared with those elicited by K^+ ions, which would affect both ganglion and trunk fibres indiscriminately. The influence of postganglionic trunk potential changes was determined by observing the responses before and after crushing the trunk. (Normally the ganglion surface was several mV positive to the postganglionic trunk immediately after excision, but this resting demarcation potential declined steadily during the course of an experiment: on crushing the postganglionic trunk the demarcation potential was restored toward its original level.) Sample records and time courses of potential changes are shown in Figs. 1 and 2 respectively.

Before the postganglionic trunk had been crushed, a ganglionic afterpositivity was observed on washing out either carbachol or K^+. However, the K^+ afterpositivity was abolished on crushing the trunk, suggesting that it resulted from delayed repolarization of the trunk. This could arise if added K^+ diffused out of the ganglion more rapidly than out of the trunk, perhaps because the connective tissue sheath had been removed from the ganglion. A slower diffusion into the postganglionic trunk would also explain the transient and declining ganglionic negativity observed on adding K^+, which was converted to a sustained negativity when the trunk was crushed. Since the degree of ' reactivity ' of the postganglionic trunk slowly increased during the course of an experiment (as judged by the declining demarcation potential) and since the relative rates of diffusion of added

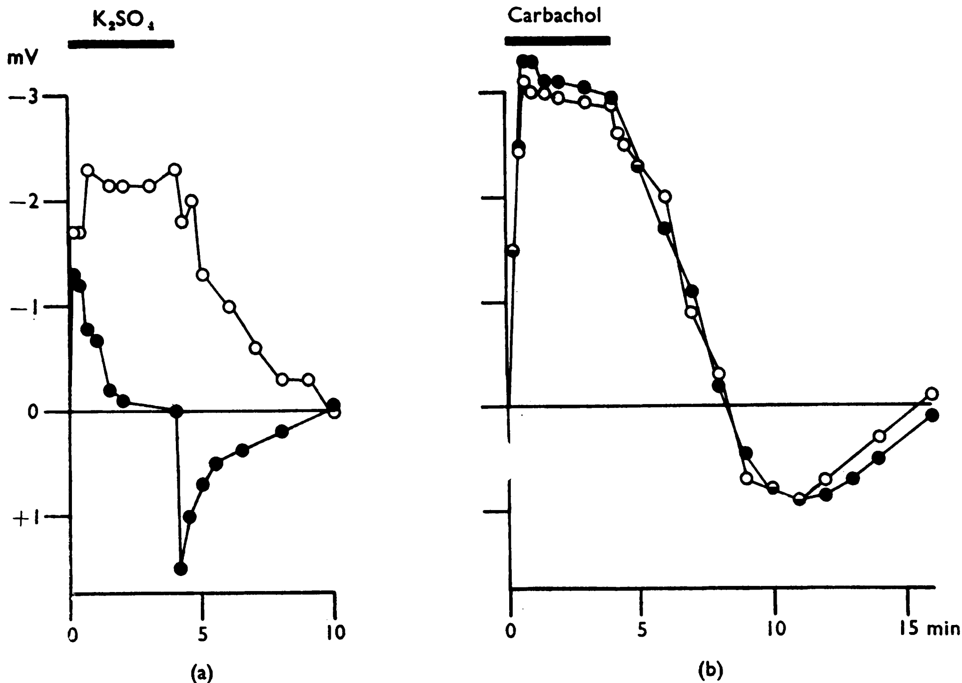


FIG. 2. Variation of surface potential with time in the experiment illustrated in Fig. 1. Ordinates: change in peak potential difference between the ganglion surface and the postganglionic trunk. Abscissae: time (min) after adding depolarizing agent to the bath fluid. The depolarizing agent (K_2SO_4 in (a), carbachol in (b)) was added for 4 min (indicated by the horizontal bar) and then washed out with normal Krebs solution. Observations were made before (●) and after (○) crushing the postganglionic nerve trunk (corresponding to (a) and (b) respectively in Fig. 1).

compounds into the ganglion and trunk might vary greatly from preparation to preparation, the moving-fluid technique is clearly unsuitable for showing the ganglionic actions of K^+ or of similar 'non-specific' depolarizing agents.

On the other hand, the sequence of potential changes produced by carbachol was quite unaffected by crushing the postganglionic nerve, showing that they arose entirely from depolarization and after-hyperpolarization of the ganglion cells. This anatomically-specific effect is also apparent from the fact that, unlike K^+, carbachol produced a sustained reversal of the resting demarcation potential (Fig. 1). Thus, the moving-fluid method gives a fairly reliable index of ganglionic potential changes produced by carbachol. Although much less than the actual membrane potential changes (because of shunting through the extracellular fluid), the measured responses were very constant during each experiment, fluctuating by $<10\%$ for a given concentration of carbachol provided that sufficient recovery time (45–60 min) was allowed between successive applications.

Post-carbachol hyperpolarization

Dose-response relationship

Both depolarization and after-hyperpolarization increased with increasing concentrations of added carbachol, though with some lack of proportionality at low levels of depolarization (Fig. 3a). Both decreased in rather similar manner in the presence of increasing concentrations of hexamethonium (Fig. 3b). Since the amount of Na^+ entering the neurones would increase as the depolarization increased, this type of relationship would be expected if the hyperpolarization resulted from the electrogenic extrusion of accumulated Na^+.

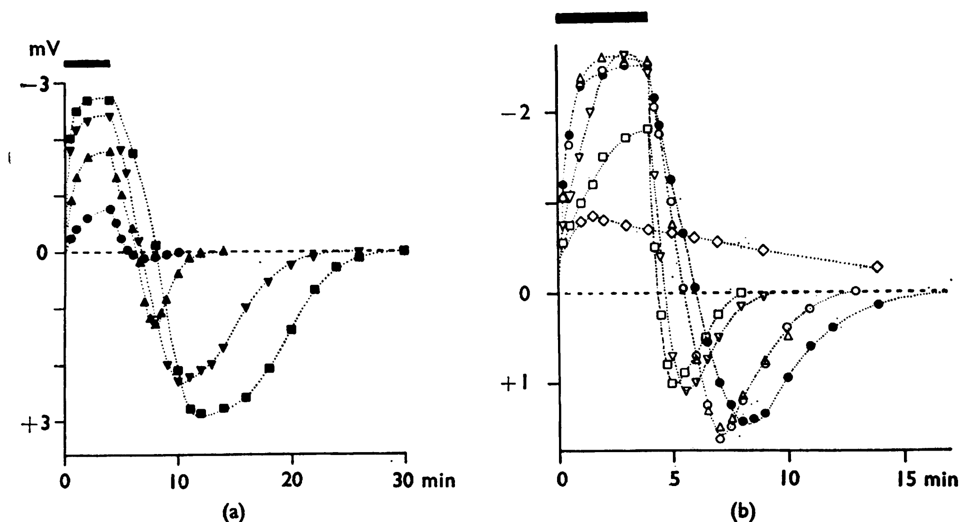


FIG. 3. Responses of two isolated rat ganglia (a) to increasing concentrations of carbachol (●, 18 μM; ▲, 55 μM; , 180 μM; ■, 550 μM) and (b) to a constant concentration of carbachol (55 μM) in the presence of increasing concentrations of hexamethonium (●, 0; ○, 2·5 μM; △, 8 μM; ▽, 25 μM; □, 80 μM; ✧, 250 μM). Carbachol was added to the bath fluid for 4 min (indicated in this and subsequent figures by the upper horizontal bar) and then washed out with fresh Krebs solution. Ordinates: ganglionic potential shift measured as in Figs. 1 & 2 (mV, ganglion depolarization −ve). Abscissae, time after adding carbachol (min). (Note the different time scales in (a) and (b).)

If the rate of Na^+ extrusion is directly proportional to the internal Na^+ concentration, the membrane hyperpolarization ($\triangle V$) would be related to the increment in internal Na^+ concentration produced by carbachol ($\triangle[Na^+]_i$) by the expression

$$\triangle V = K.\frac{(1-C)}{G_m}.\triangle[Na^+]_i e^{-kt}$$

where K is a proportionality constant, C is the coupling ratio between K^+-uptake and Na^+ extrusion, G_m is the membrane conductance and k is a rate constant (*cf.* Rang & Ritchie, 1968). Thus, provided that the coupling ratio and membrane conductance remain constant, the after-hyperpolarization should decline exponentially with time, with a rate-constant k. Also, the maximum hyperpolarization ($\triangle V_{max}$) should be directly proportional to the initial increment in internal Na^+ concentration and the area under the hyperpolarization-time curve ($\int \triangle V.dt$) should be proportional to the total amount of Na^+ extruded.

In practice, the time-course deviated markedly from an exponential during its early phase (Fig. 4c). Since the coupling ratio is probably constant (Thomas, 1969), this can most reasonably be attributed to a varying membrane conductance. One likely cause of this is that carbachol may diffuse out of the ganglion quite slowly on changing the bath fluid, so that a residual effect on the receptors may elevate the membrane conductance above the resting level for some time after washing. In this case, adding a receptor-blocking agent to the washout fluid should reduce the membrane conductance more rapidly. Fig. 4 shows the effect of adding hexamethonium to the washout fluid: the maximum amplitude of the hyperpolarization was increased and the onset of hyperpolarization accelerated (Fig. 4a). The latter probably results from the fact that, in the absence of hexamethonium, Na^+ continues to enter the cells for some minutes after washing, and hexamethonium hastens the initial decline in the internal Na^+ concentration (C. N. Scholfield, un-

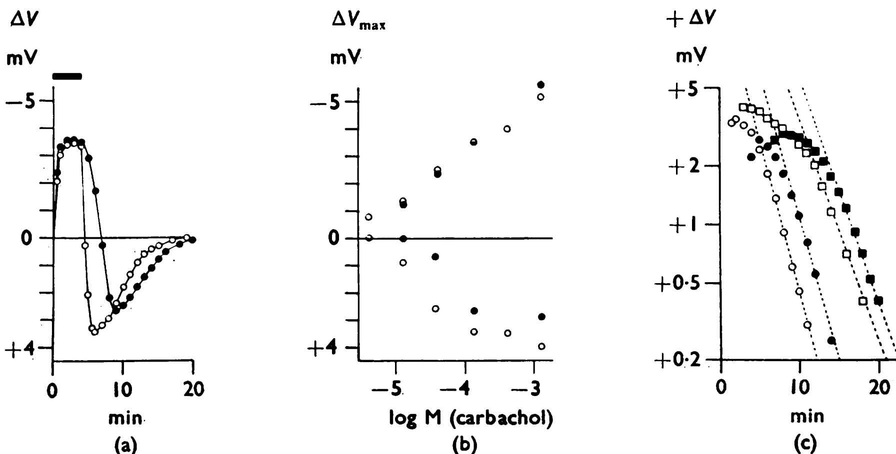


FIG. 4. Post-carbachol hyperpolarization recorded on washing the ganglion with normal Krebs solution (filled symbols) and with Krebs solution containing 2·5 mM hexamethonium (open symbols). (a) Time-course of the potential changes plotted as in Figs. 2 & 3. (b) Maximum depolarization (−) and after-hyperpolarization (+) plotted against log molar carbachol concentration. (c) Semi-logarithmic plot of the after-hyperpolarization alone (+$\triangle V$) versus time after washout for two concentrations of carbachol 110 μM (●, ○) and 1·1 mM (■, □) in the absence (●, ■) and presence (○, □) of hexamethonium.

published observations). Continued Na^+ entry might also explain the increase, with increasing concentrations of carbachol, in the time taken for the hyperpolarization to reach a peak (Fig. 3a).

Hexamethonium did not affect the final rate constant, suggesting that the membrane conductance had returned to its resting level and was indeed constant during the exponential phase (Fig. 4c). In this case, the rate constant becomes a reasonable measure of the rate of Na^+ extrusion. In 17 ganglia exposed to 110 or 180 μM carbachol at temperatures of 22° to 27° C, the mean rate constant ($\pm$S.E. of mean) was $0{\cdot}38 \pm 0{\cdot}02$ min^{-1}. The rate constant was not consistently affected by varying the carbachol concentration over the range 110 to 580 μM (3 experiments), although fluctuations in the course of an experiment occurred as in Fig. 4. Measurements at carbachol concentration below 100 μM were impracticable because the hyperpolarization was then very small.

Metabolic dependence

In this and most subsequent parts of the investigation, the effects of various procedures which would be expected to affect Na^+ extrusion were tested on the after-hyperpolarization that followed a constant dose of carbachol (usually 180 μM). The carbachol was applied for 4 min at intervals of 45–60 minutes. In early experiments the washout fluid was normal Krebs solution. Later, when the significance of the post-carbachol conductance increase became apparent, hexamethonium (2·5 mM) was routinely added at washout (see above). The hexamethonium solution was then replaced by normal Krebs solution at the end of hyperpolarization, and at least 20 min before the next carbachol application. This was sufficient to prevent any depression of subsequent carbachol depolarization.

Effect of temperature change. Hyperpolarization due to active Na^+ extrusion should show a greater sensitivity to temperature than depolarization resulting from passive ionic movements. Pascoe (1956) observed that the hyperpolarization was selectively reduced on cooling the ganglion below 30° C. We have confirmed this observation: Fig. 5a shows that cooling from 27° C to 8° C reduced the after-hyperpolarization without a corresponding effect on the preceding depolarization. When the ganglion was re-warmed, a secondary hyperpolarization occurred. Since warming did not produce such an effect in the absence of added carbachol, this secondary hyperpolarization may be attributed to re-activation of the Na^+ pump at a time when the intracellular Na^+ concentration was still raised (*cf.* Rang & Ritchie, 1968).

Rather oddly, warming the bath fluid above 30° C also reduced the after-hyperpolarization (Fig. 5b). This seems inconsistent with a metabolically-driven process. It might be explained if the Na^+ pump were so accelerated that most of the Na^+ was extruded during the period when the membrane conductance was raised by the residual action of the carbachol, such that the electrogenic effect of Na^+ extrusion was dissipated by passive ionic movements. In support of this hypothesis, the depression of hyperpolarization at raised temperatures was prevented by adding hexamethonium to the washout fluid (fourth response in Fig. 5b).

Progressive effects of temperature change on the maximum amplitude of carbachol depolarization ($-\Delta V_{max}$) and on three parameters of the after-hyperpolarization, maximum amplitude ($+\Delta V_{max}$), time to half-decay ($t_{0{\cdot}5}$) and area under the

hyperpolarization-time curve ($\int + \Delta V.dt$, measured by planimetry), are shown in Fig. 6. (The time to half-decay or 'half-time", being measured from the time of peak hyperpolarization, is not a true measure of the rate of Na^+ extrusion, since the time-course was not usually exponential in this region (see Fig. 4c). However, the exponential time-constant could not be measured over the entire temperature range, since the hyperpolarization was too small at low temperatures. In practice, changes in the half-time were fairly similar to changes in the exponential time-constant when measured over narrow temperature ranges.)

Lowering the temperature to 10° C had little effect on the depolarization perhaps because any reduction of the conductance increase produced by carbachol may be balanced by a fall in resting conductance. The reduced amplitude and lengthened

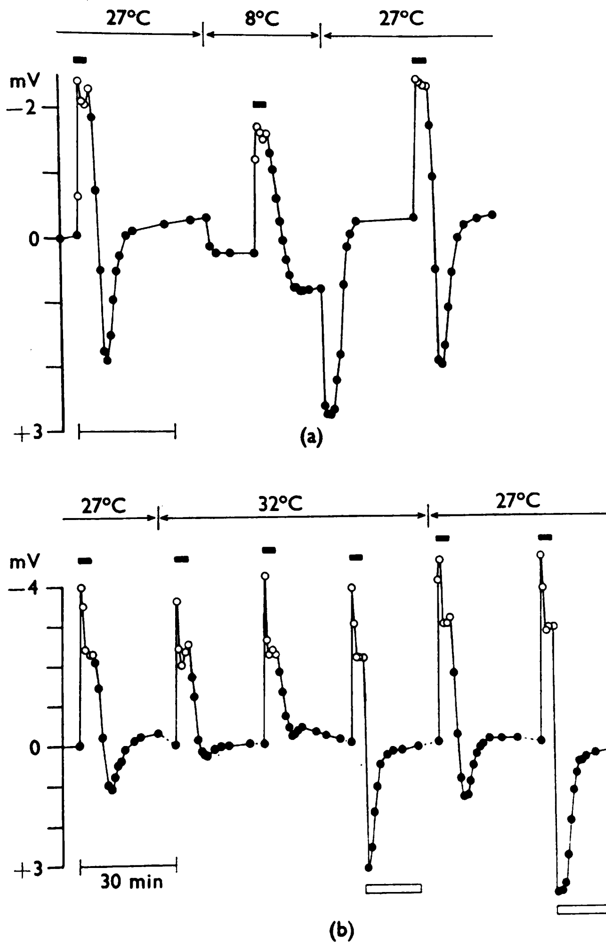


FIG. 5. Effects of (a) cooling and (b) warming from ambient temperature (27° C) on ganglion depolarization and after-hyperpolarization produced by 4 min application of 180 μM carbachol (two experiments). In this and Figs. 7–11, changes in the potential difference between the ganglion surface and the postganglionic trunk are plotted continuously against time (ganglion depolarization upwards), the resting potential at the start being arbitrarily set at zero. The thick horizontal bars above the records indicate the addition of carbachol and measurements made during exposure to carbachol are shown by the open circles. Interrupted lines between points indicate breaks of about 15 min. Following the fourth and sixth doses of carbachol in (b), hexamethonium (2·5 mM) was added to the washout fluid for the duration indicated by open bars below the records.

time-course of the hyperpolarization may be attributed to a reduced rate of Na^+ extrusion. The amplitude would be affected by any reduced membrane conductance which would tend to counteract the effect of cooling on the Na^+ pump by increasing its electrogenic efficiency. An increased electrogenic effect would also explain the increased area of hyperpolarization: since the latter reflects the total amount of Na^+ extruded, if the membrane conductance remained constant. the area would be expected to decline on cooling as the Na^+ influx was reduced.

The Q_{10} for Na^+ extrusion in this tissue, as judged from the 'half-time' measured in the presence of hexamethonium, was about 2·2. In one experiment where a large (5 mV) hyperpolarization was recorded at room temperature, the true exponential rate constant could be measured quite accurately, and showed a Q_{10} of 2·3 between 17° C and 27° C. These values accord quite well with the Q_{10} for electrogenic Na^+ extrusion measured in vagal fibres (2·6 between 16° C and 26° C: den Hertog & Ritchie, 1969) and in spinal neurones (2·3 between 30° C and 40° C: Kuno *et al.*, 1970).

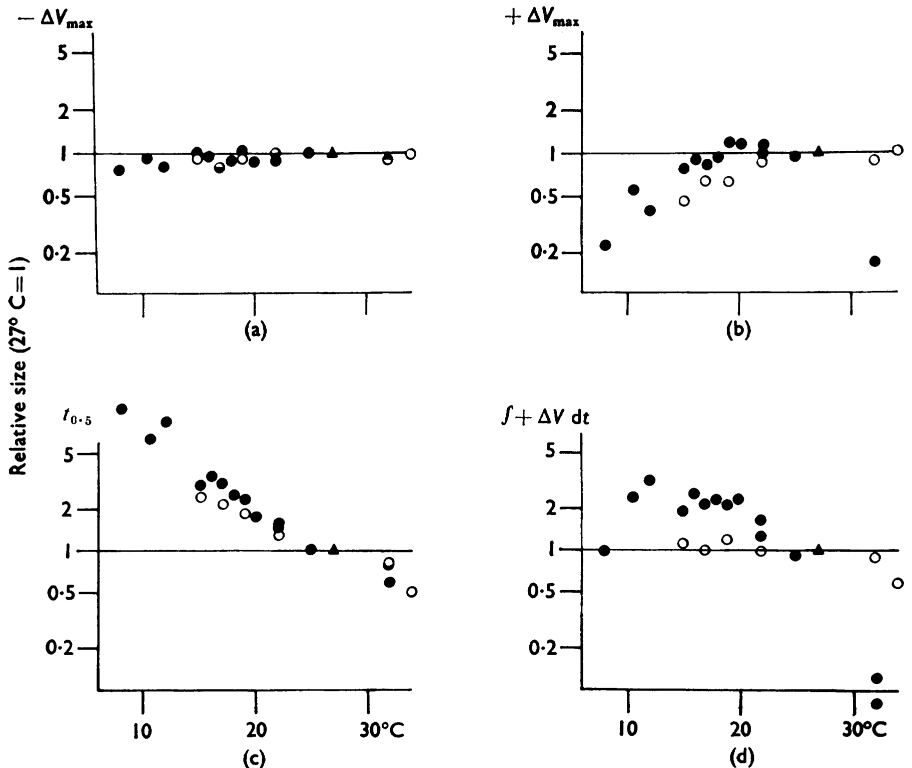


FIG. 6. Effects of varying the bath temperature on: (a) maximal amplitude of ganglion depolarization ($-\Delta V_{max}$) produced by 180 μM carbachol; (b) maximal amplitude of ganglionic after-hyperpolarization ($+\Delta V_{max}$); (c) time to half-decay of the after-hyperpolarization ($t_{0\cdot5}$); and (d) area under the hyperpolarization-time curve ($\int + \Delta V.dt$). Each is expressed as a fraction of the response observed at 27° C (indicated by ▲). Each point represents a single observation, made either in normal Krebs solution (●) or with hexamethonium (2·5 μM) added to the washout fluid (○) and were obtained from a total of six experiments.

Action of metabolic inhibitors. The after-hyperpolarization could be abolished by metabolic inhibitors such as sodium cyanide (2 mM), sodium azide (0·5 to 2 mM) or 2,4-dinitrophenol (DNP, 0·2 to 1 mM) without any substantial reduction of the preceding depolarization (Fig. 7). On washing out the inhibitor, the hyperpolarization could be regenerated just as with cooling and subsequent re-warming. The effects of lower concentrations of inhibitors was difficult to quantitate, but appeared qualitatively similar to those described in non-myelinated fibre trunks by Ritchie & Straub (1957) and by den Hertog & Ritchie (1969).

Influence of the external K^+ concentration. Electrogenic Na^+ extrusion, like the coupled Na^+ pump, is slowed when K^+ is omitted from the external medium, and is then resumed when K^+ is restored, so that a secondary peak of hyperpolarization occurs (Kerkut & Thomas, 1965; Rang & Ritchie, 1968). Such effects have also been reported for post-acetylcholine hyperpolarization in rabbit ganglia (Kosterlitz *et al.*, 1970) and are illustrated for rat ganglia in Fig. 8a. Unlike the addition of metabolic inhibitors (Fig. 7) or ouabain (Fig. 9) omission of K^+ did not completely prevent the hyperpolarization. The reason may be that K^+ continues to leak out of the cells sufficiently fast to maintain an appreciable perineuronal concentration (*cf.* Hodgkin & Keynes, 1955). The K_m for K^+ activation of ganglionic hyperpolarization is quite low (about 3 mM, see here), and slow exchange between interstitial fluid and bath fluid would favour retention of K^+ in the interstitial spaces. In addition the electrogenic effect of the Na^+ pump might be increased at low K^+ concentrations, since this ion may carry some of the leak current (see p. 668).

When K^+ concentration of the bathing fluid was increased progressively from 0 to 40 mM $[K^+]_o$ (by adding KCl to K^+ free solution) the peak amplitude of the hyperpolarization increased up to 10 mM $[K^+]_o$ and the half-time was reduced up to 20 mM $[K^+]_o$. This may be attributed principally to an accelerated rate of Na^+

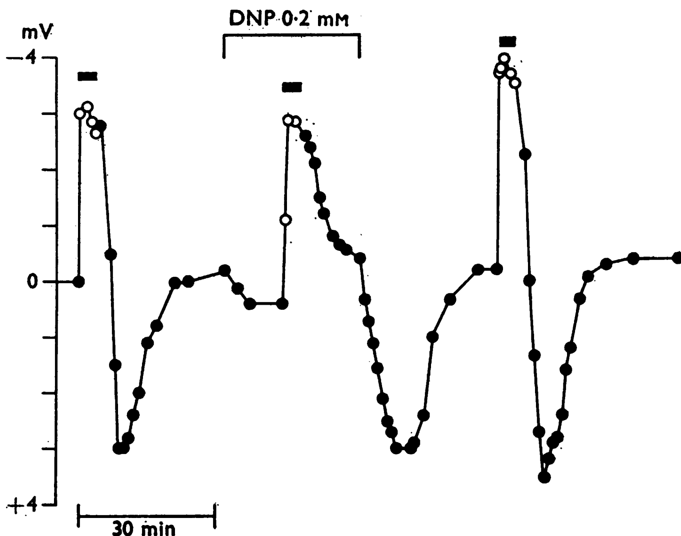


FIG. 7. Effect of 2,4-dinitrophenol (DNP; 0·2 mM) on ganglion depolarization and after-hyperpolarization produced by 180 μM carbachol, plotted on a continuous time-scale as in Fig. 6.

extrusion, the effects being complicated by the increased membrane conductance. The apparent K_m for K^+ activation of electrogenic Na^+ extrusion in this tissue, judged from the change in 'half-time' and rate constant (see Fig. 9) was about 3–4 mM, i.e. quite similar to that for electrogenic hyperpolarization in vagal fibres (2·8 mM, *cf.* Rang & Ritchie, 1968).

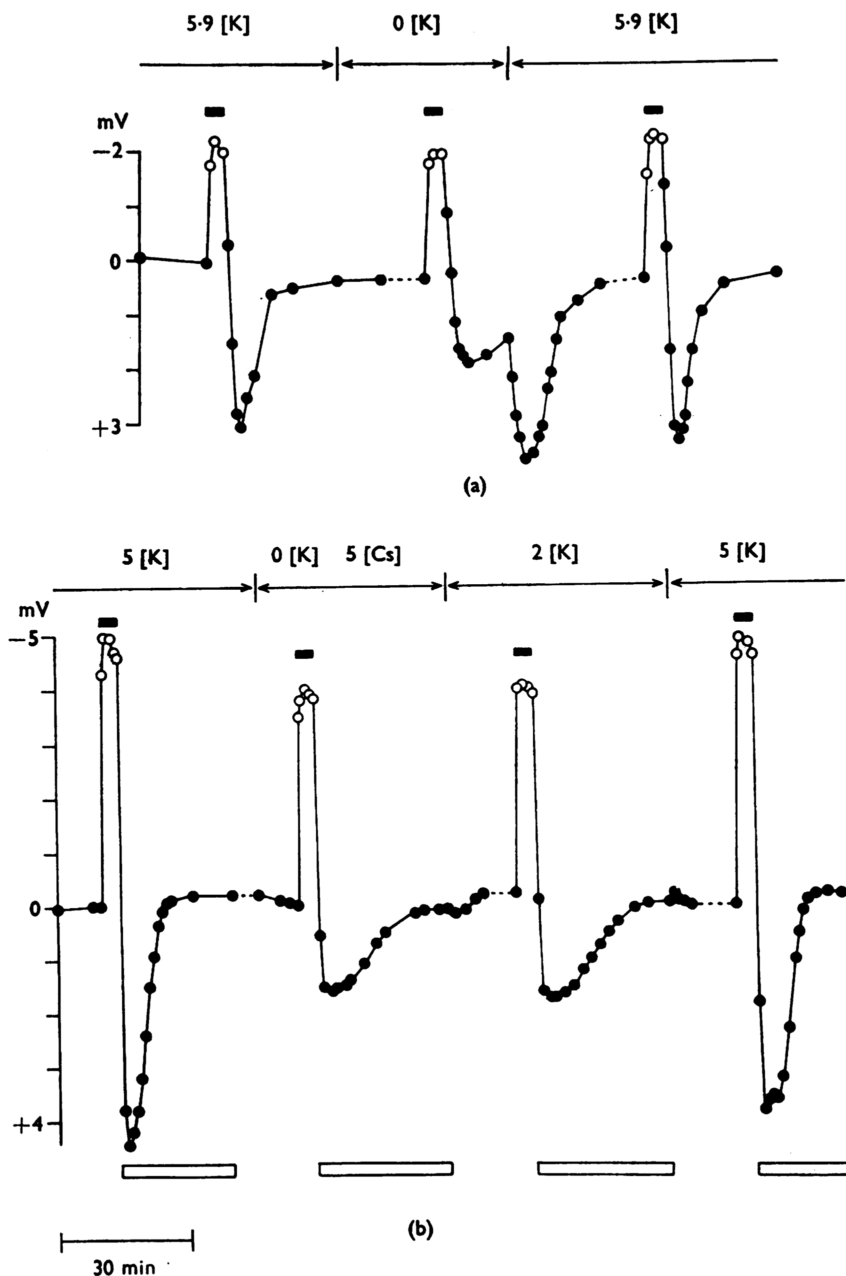
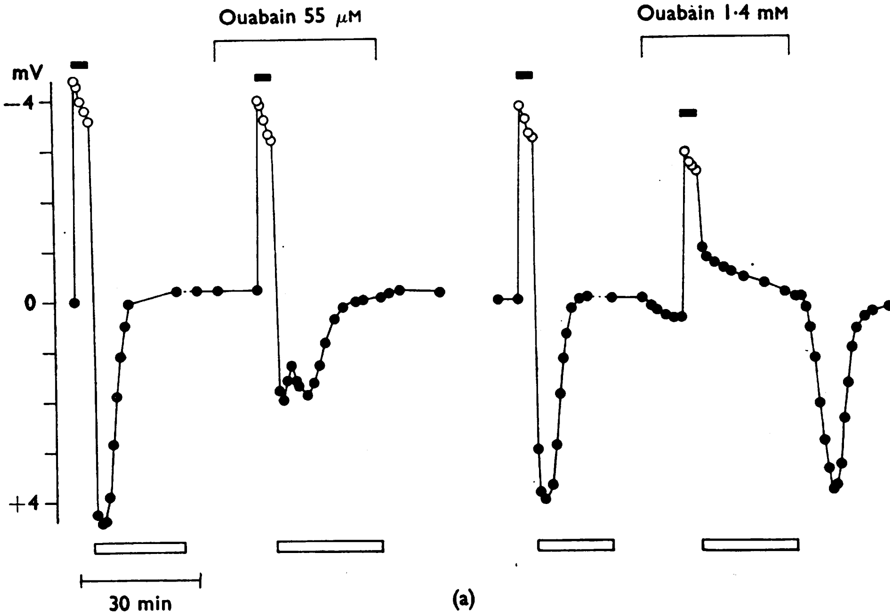


FIG. 8. Effects of (a) omitting K^+ (0 [K]) from normal Krebs solution (5·9 [K]) and (b) reducing $[K^+]_o$ from 5 to 2 mM or substituting 5 mM Cs^+ for K^+ (0 [K] 5 [Cs]) on carbachol-depolarization and post-carbachol hyperpolarization (2 experiments).

Caesium and rubidium ions. Substitution of Cs^+ for K^+ reduced postcarbachol hyperpolarization: the effect of 5 mM [Cs^+] was approximately equivalent to 2 mM [K^+] (Fig. 8b). Substitution of Rb^+ for K^+ also reduced the hyperpolarization, but by a smaller amount than Cs^+. Thus, the relative activities of these three



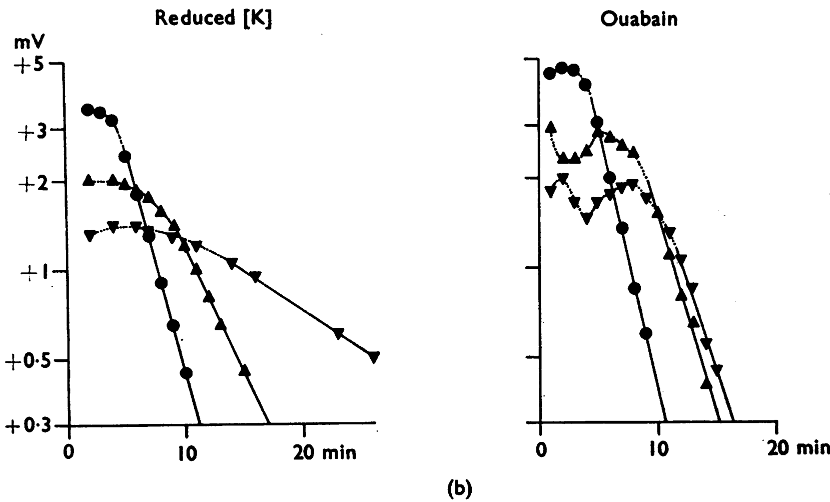


FIG. 9. (a) Effects of ouabain (55 μM and 1·4 mM) on carbachol depolarization and after-hyperpolarization, recorded from a single preparation but with an interval of several hours between the two tests. (b) Comparison of the effects of reducing $[K^+]_o$ (left side) and adding ouabain (right side) on the time-course of post-carbachol hyperpolarization, plotted semi-logarithmically as in Fig. 4c. Symbols in (b): left side, ○ 5 mM, ▲ 2 mM and ▼ 0·5 mM $[K^+]_o$; right side, ● 0, ▲ 27 and ▼ 55 μM ouabain. These effects of $[K^+]_o$ and ouabain were recorded from different preparations, but were representative of several other experiments.

cations ($K^+ \gg Rb^+ > Cs^+$) in supporting post-carbachol hyperpolarization is qualitatively similar to their relative effects as activators of Na-K ATPase (Skou, 1960), of Na^+ extrusion from crab nerves (Baker & Connelly, 1966), and of the electrogenic Na^+ pump in vagus nerve fibres (Rang & Ritchie, 1968).

Effects of ouabain. The stimulation of Na^+ extrusion by K^+ results from activation of Na-K ATPase, and this activation is inhibited by cardiac glycosides (Glynn, 1957, 1968 ; Skou, 1965). Consequently ouabain should block the ganglion after-hyperpolarization. This was true to the extent that ouabain depressed the hyperpolarization in concentrations which did not affect the preceding depolarization (Fig. 9a ; *cf.* Kosterlitz *et al.*, 1970). A rather high concentration of ouabain was needed (about 40 μM for 50% reduction of ΔV_{max}) but inhibition of Na-K ATPase in other rat tissue requires a similar concentration of ouabain (Aldridge, 1962 ; Akera, Larson & Brody, 1969). However, there were some differences between the effects of ouabain and of low $[K^+]_o$. (i) In sufficient concentrations ($\sim$1 mM), ouabain completely annulled the hyperpolarization, which was then regenerated on washing out the ouabain (Fig. 9a). The reason for incomplete blockade with zero $[K^+]_o$ is discussed above. (ii) With intermediate ouabain concentrations, two peaks of hyperpolarization were often seen (Fig. 9a). This was not observed with low $[K^+]_o$ or with metabolic inhibitors. In view of the interaction of ouabain and $[K^+]_o$ (Glynn, 1957 ; den Hertog & Ritchie, 1969) it might result from fluctuations in perineuronal $[K^+]_o$ with changing pumping rates. (iii) Relative to the reduction in its amplitude, the time-course of hyperpolarization was rather little affected by ouabain (Fig. 9b), so that the area of the hyperpolarization was usually reduced. Similar effects of ouabain on electrogenic hyperpolarization in vagus nerves has been observed, and attributed to mixed stimulant and depressant actions, or to variable depressant actions, on different populations of fibres (den Hertog & Ritchie, 1969).

Mersalyl. At a concentration of 0·2 to 0·4 mM, mersalyl reduced post-carbachol hyperpolarization with little effect on depolarization. The action of mersalyl was not easily reversed by washing. Reversal was accelerated by adding 0·5 mM 2,3-dimercaprol (BAL) which led to a partial regeneration of the hyperpolarization. This indicates that the effect of mersalyl results from —SH binding. Another —SH binding agent, p-choloromercuribenzoate, was tested for a comparable action, but reduced both depolarization and after-hyperpolarization in a non-selective manner.

Effect of lithium ions

Li^+ is not an effective substrate for the Na^+ pump (Keynes & Swan, 1959 ; Baker & Connelly, 1966 ; Wespi, 1968), so that an influx of Li^+ instead of Na^+ into the neurone will not lead to an electrogenic hyperpolarization (Ritchie & Straub, 1957 ; Kerkut & Thomas, 1965 ; Rang & Ritchie, 1968). When Na^+ was largely (118 mM) or completely (143 mM) replaced by Li^+ no after-hyperpolarization of the rat ganglion occurred following application of carbachol (Fig. 10). Li^+ substitution also reduced the depolarization produced by carbachol, perhaps because the permeability to Li^+ is less than that to Na^+ (Armett & Ritchie, 1963 ; Wespi, 1968). This could sometimes be countered by raising the concentration of carbachol, still without an after-hyperpolarization. Li^+ also inhibits the activation of the Na^+ pump by external K^+ (Baker & Connelly, 1966). This does not account for the lack of after-hyperpolarization in Li^+ solution, since replacement

by Li^+ immediately after the depolarization instead of beforehand increased the hyperpolarization (perhaps because of a reduced leak current). Consequently, the effect of Li^+ can be attributed to a reduced entry of Na^+ and indicates a specific requirement for intracellular Na^+ ions in generating the after-hyperpolarization.

Effect of calcium

Kosterlitz *et al.* (1970) have reported that post-acetylcholine hyperpolarization in rabbit ganglia is reduced by omitting Ca^{2+} from the bathing fluid. This also occurred in rat ganglia (Fig. 11): conversely increasing $[Ca^{2+}]_o$ from 2·5 to 10 mM slightly augmented the after-hyperpolarization.

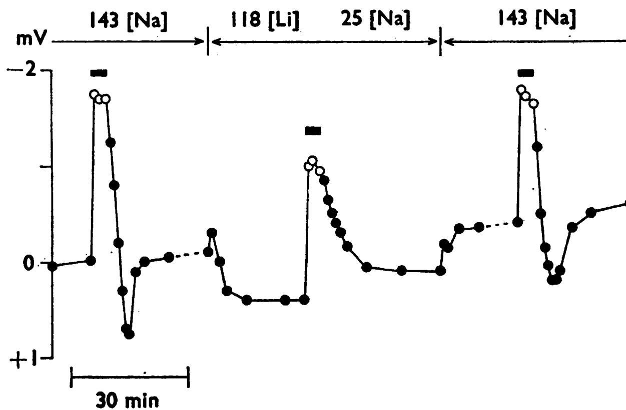


FIG. 10. Abolition of post-carbachol hyperpolarization by partly replacing Na^+ (143 mM in normal Krebs solution) with Li^+ (118 mM $[Li^+]$ as LiCl, 25 mM $[Na^+]$ as $NaHCO_3$).

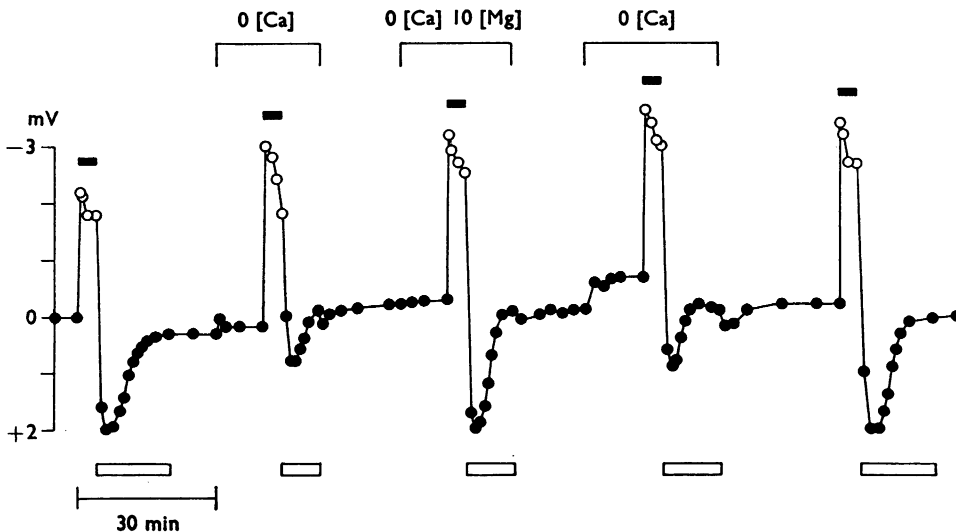


FIG. 11. Reduction of post-carbachol hyperpolarization on omitting Ca^{2+} from the bathing fluid (0 [Ca]) and its reversal by raising Mg^{2+} to 10 mM (0 [Ca] 10 [Mg]). Normal Krebs solution contained 2·5 mM $[Ca^{2+}]$ and 1·2 mM $[Mg^{2+}]$.

This effect is rather curious since Ca^{2+} inhibits Na-K ATPase (Skou, 1960). Baker, Blaustein, Hodgkin & Steinhardt (1969) have described a form of Na^+ pumping in squid axons which is activated by Ca^{2+} but not by Mg^{2+}, and inhibited by La^{3+}. This does not account for the Ca^{2+} effect on ganglion after-hyperpolarization since the latter was unaffected by 1 mM [La^{3+}] and Mg^{2+} could substitute for Ca^{2+} (Fig. 11). The most probable explanation is that the membrane conductance was increased in the abstract of Ca^{2+} and reduced again when [Mg^{2+}] was added (*cf.* Frankenhaeuser & Hodgkin, 1957). This would accord with the lack of any obvious effect of Ca^{2+} free solution on the time-course of the hyperpolarization.

Role of Chloride ions

In vagus fibres the membrane hyperpolarization produced by the electrogenic Na^+ current is normally reduced by a very considerable passive efflux of Cl^-: thus, when external Cl^- is replaced by some impermeant anion such as isethionate post-tetanic hyperpolarization is gradually augmented as the internal Cl^- concentration falls (Rang & Ritchie, 1968). This type of effect was not seen very clearly in the isolated rat ganglion nor has it been observed in the rabbit ganglion (Lees & Wallis, personal communication): changes in both depolarization and after-hyperpolarization following substitution of isethionate for Cl^- were variable but rather minor (Table 1). This suggests that Cl^- movements do not contribute to the depolarization and do not greatly affect the hyperpolarization produced by the Na^+ pump.

Effect of atropine and hyoscine

Some forms of hyperpolarization appear to be mediated through stimulation of 'muscarinic' receptors (Volle, 1966; Koketsu, 1969; Pinsker & Kandel, 1969). Since carbachol can stimulate 'muscarinic' receptors in ganglia (Brown, 1966b), the after-hyperpolarization might involve a component of delayed 'muscarinic' activity. This seems rather improbable since (among other reasons) the hyperpolarization was abolished when the depolarization was reduced by hexamethonium (Fig. 3b). As a further check the effects of atropine and hyoscine were tested. Neither atropine (up to 15 μM) nor hyoscine (up to 26 μM) affected the after-

TABLE 1. *Effect of substituting isethionate for chloride on ganglion depolarization* ($-\Delta V$) *and after-hyperpolarization* ($+\Delta V$) *produced by* 0.18 mM *carbachol. Responses are expressed as a fraction of those observed in normal Krebs solution.*

Expt.	Depolarization	After hyperpolarization			
	$-\Delta V$max	$+\Delta V$max	$\int +\Delta V.dt$	$t_{0\cdot5}$	k
1	1·04	1·43	1·78	1·24	0·87
*1	1·26	1·32	1·16	0·78	0·92
2	1·18	0·91	1·03	0·96	1·18
*2	1·30	1·03	0·84	0·90	1·21
*3	0·90	1·22	1·45	1·22	1·05
*4	1·16	1·10	1·29	1·11	—
mean	1·14	1·17	1·26	1·04	1·05
±S.E.	±0·06	±0·08	±0·13	±0·08	±0·07

* With hexamethonium 2·5 mM added to the washout fluid.

hyperpolarization. These concentrations were too low to materially alter carbachol-depolarization, but are at least 10 times those required to block the action of muscarine on the ganglion (Brown, 1966b).

Effect of tetrodotoxin

Na^+ might enter the ganglion cells during application of carbachol both as a direct effect of receptor activation and also because the depolarization generates action potentials. The latter appears not to be an important cause of the Na^+ influx because the after-hyperpolarization was not reduced by tetrodotoxin in a concentration (0·6 μM) which abolished conducted and transmitted action potentials.

Hyperpolarization after other depolarizing agents

Acetylcholine. Hyperpolarization of isolated rat ganglia following acetylcholine-depolarization has been reported previously (Pascoe, 1956; Brown, 1966a). The rate-constant for post-acetylcholine hyperpolarization ($0{\cdot}40 \pm 0{\cdot}01$ min^{-1}, $n=18$) was similar to that for post-carbachol hyperpolarization. However, for the same amount of depolarization the rate of onset of post-acetylcholine hyperpolarization was faster and the peak amplitude greater (see Fig. 2 in Brown, 1966a). An obvious explanation for this might be that the conductance increase produced by acetylcholine is terminated more rapidly by the action of cholinesterase. However, anticholinesterase agents did not clearly delay the onset of the hyperpolarization or reduce its amplitude. One problem with acetylcholine is that the concentration required to depolarize the ganglion in the absence of an anticholinesterase is very high (1 mM or more) so that some of its depolarizing action might be mediated through the formation of choline (see Kosterlitz *et al.*, 1968). If so, the action of acetylcholine is likely to be more complex than that of carbachol, and the effect of anticholinesterase agents rather unpredictable.

Nicotine. No after-hyperpolarization occurred when the depolarizing action of nicotine was terminated by washing with normal Krebs solution: it could only be recorded by adding hexamethonium to the washout fluid. The hyperpolarization remained somewhat smaller than that seen after carbachol, but had a similar time-course. Presumably the membrane conductance remained sufficiently high on washing with normal Krebs solution to offset completely the electrogenic effect of Na^+ extrusion. Cl^- did not contribute significantly to this high leak current, since replacement of Cl^- with isethionate in the absence of hexamethonium did not lead to an after-hyperpolarization.

Sodium ethylenediaminetetracetate, EDTA. Application of 1 to 2 mM EDTA in Ca^{2+}-free solution produced a ganglionic negativity which was also followed by a pronounced after-positivity on washing. The after-positivity seemed to result from ganglionic hyperpolarization rather than changes in trunk potential (*cf.* K^+ ions, Fig. 2) since (*a*) its time-course was prolonged like that following wash-out of carbachol, (*b*) it persisted when the postganglionic trunk was crushed, and (*c*) it was selectively reduced by metabolic inhibitors. These concentrations of EDTA produce an influx of Na^+ in the isolated ganglion (C. N. Scholfield, unpublished observations), and the after-hyperpolarization may be a consequence of this.

Discussion

The hyperpolarization of the isolated rat ganglion following depolarization by carbachol is clearly dependent upon the rate of active Na^+ extrusion. Thus, it is strongly reduced or abolished by metabolic inhibition, by inhibiting Na-K ATPase activity, or by replacing Na^+ with Li^+. Two explanations for this dependence seem possible. Firstly, the hyperpolarization might be generated directly by an electrogenic Na^+ pump; or secondly, it might be due to an increased K^+ permeability (P_K), the effect of the Na^+ pump being to maintain a high value for the K^+ equilibrium potential (E_K). The strongest argument against the latter is that pointed out by Rang & Ritchie (1968), namely that if the membrane potential were determined by E_K then restoring K^+ to a K^+-free solution during the hyperpolarization should depolarize the ganglion; whereas in fact, the reverse occurred (Fig. 8), a result which is difficult to explain on any other basis than activation of an electrogenic pump. The regeneration of a hyperpolarization on removing other forms of Na^+ pump inhibition may be interpreted along similar lines. However, this effect does not form such a strong argument for an electrogenic pump as that of K^+ since it could be explained by rapid changes in perineuronal K^+ concentration (*cf.* Ritchie & Straub, 1957).

Comparison with post-tetanic hyperpolarization

In most of its properties, post-carbachol hyperpolarization is very similar to post-tetanic hyperpolarization in unmyelinated nerve fibres (*cf.* Ritchie & Straub, 1957; Rang & Ritchie, 1968; den Hertog & Ritchie, 1969). Two differences emerge from the present observations, concerning respectively the nature of the leak current and the apparent kinetics of Na^+ extrusion.

Leak current. In vagal fibres the voltage change produced by the Na^+ current is dissipated to a large extent by a passive efflux of Cl^- ions, so that the hyperpolarization is greatly enhanced on replacing Cl^- with an impermeant anion (Rang & Ritchie, 1968). This did not happen with the ganglionic after-hyperpolarization, suggesting that any leak currents are carried by cations. One reason for this is that the resting membrane permeability of sympathetic ganglion cells to Cl^- seems to be much lower than that of vagal fibres. Thus, in the rabbit ganglion the permeability ratio $P_K:P_{Cl}$ has been calculated from the ionic concentrations and resting membrane potential to be about 1:0·02 (Woodward, Bianchi & Erulkar, 1969), whereas a similar calculation applied to the data of Armett & Ritchie (1963) and Keynes & Ritchie (1965) suggests the corresponding ratio for vagal fibres to be 1:0·45. The rates of ^{42}K efflux from the two tissues are fairly similar (*cf.* Harris & McLennan, 1953, and Keynes & Ritchie, 1965) so these different permeability ratios would suggest a twenty-fold difference in Cl^- permeabilities. A second factor which would lead to a high cationic permeability in the ganglion is that the leak current during the early phase of post-carbachol hyperpolarization can be largely attributed to a residual effect of carbachol on the receptors which would involve a selective increase in cationic permeability relative to that for anions (Jenkinson & Nicholls, 1961; Takeuchi, 1963; Koketsu, 1969).

Kinetics of Na^+ extrusion. The time-course of electrogenic hyperpolarization in vagal fibres could be interpreted in terms of a Na^+ extrusion process whose rate was a saturable function of the third power of $[Na^+]_i$ (Rang & Ritchie, 1968). Maximal hyperpolarization of the vagus was attained after stimulation for 5 s at

30 Hz. Assuming the Na^+ influx to equal K^+ efflux during stimulation (about (0·02 mmoles/kg cell water)/impulse, Keynes & Ritchie, 1965), the Na^+ pump would have been saturated when $[Na^+]_i$ was about 3 mM. On the other hand, Brinley & Mullins (1968) found that the rate of Na^+ extrusion in squid axons was directly proportional to $[Na^+]_i$ up to concentrations as high as 240 mM. In the ganglion, $[Na^+]_i$ may increase by up to 50 mM after 4 min exposure to carbachol (C. N. Scholfield, unpublished observations), and the exponential recovery kinetics suggest that, as in the squid axons, Na^+ extrusion is directly proportional to $[Na^+]_i$ up to quite high concentrations of Na^+.

Ganglion hyperpolarization in vivo

Depolarization of cat superior cervical ganglia *in vivo* by stimulant agents is followed by a ganglion hyperpolarization, distinguished from the muscarinic-type of hyperpolarization (*cf.* Takeshige, Pappano, de Groat & Volle, 1963; Takeshige & Volle, 1964) by its appearance after the injection of nicotinic stimulants and by its resistance to atropine (Brown, 1966c; Gebber & Volle, 1966). This non-muscarinic hyperpolarization might also be due to electrogenic Na^+ extrusion since it is reduced by ouabain (Gebber & Volle, 1966) and, in a perfused ganglion, by replacing Na^+ with Li^+ (Jaramillo & Volle, 1968). It was also found that injection of Cs^+ ions increased the amplitude of the hyperpolarization (Hancock & Volle, 1969) which may be explained by the activation of the Na^+ pump by Cs^+. The hyperpolarization *in vivo* occurs after only a few seconds' depolarization, showing that prolonged drug application is not an essential requirement; presumably, much less Na^+ entered the ganglion than *in vitro*, but the rate of Na^+ extrusion would be higher *in vivo* because of the higher temperature. In the experiments on cat ganglia, the after-hyperpolarization following tetramethylammonium injections was accompanied by a partial blockade of ganglionic transmission, which was alleviated by injecting ouabain (Gebber & Volle, 1966). Thus, the electrogenic after-hyperpolarization might contribute to the ganglion-blockade produced by depolarizing agents.

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