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SEROTONIN DEPRESSES THE AFTER-HYPERPOLARIZATION THROUGH THE INHIBITION OF THE Na^+/K^+ ELECTROGENIC PUMP IN T SENSORY NEURONES OF THE LEECH

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Summary

In T sensory neurones of the leech, a train of impulses elicited by intracellular electrical stimulation leads to an after-hyperpolarization of up to 30 mV, mainly due to the activation of the electrogenic Na^+/K^+-ATPase but partly to a Ca^{2+}-activated K^+ conductance. It was found that serotonin reversibly reduced the amplitude of this after-hyperpolarization. We investigated the mechanism of action of serotonin and found: (1) after inhibition of the Ca^{2+}-activated K^+ conductance with $BaCl_2$ or $CdCl_2$, serotonin was still able to reduce the after-hyperpolarization; (2) when penetration of T cells with microelectrodes leaking sodium was preceded by serotonin perfusion of the ganglia, the normal hyperpolarization due to the activation of the electrogenic pump was converted to a depolarization; (3) after long-lasting perfusion with K^+-free saline solution (which inhibits the Na^+/K^+ pump), the application of CsCl caused repolarization by reactivating the electrogenic ATPase; serotonin slowed and reduced this repolarization; (4) serotonin potentiated the depolarization of T neurones caused by the inhibition of the Na^+/K^+ pump following cooling of ganglia and depressed the hyperpolarization after rewarming to room temperature. These data taken together suggest that serotonin directly inhibits the Na^+/K^+ electrogenic pump.

Introduction

In each segmental ganglion of the leech nervous system, three types of mechanosensory neurones have been identified: touch (T), pressure (P) and nociceptive (N) cells (Nicholls and Baylor, 1968). The firing discharge of these neurones produces an after-hyperpolarization (AHP) which is involved in important physiological functions: it leads to the inhibition of the previously activated pathway, to an elevation of the volley threshold, to a decrease of synaptic efficacy

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at the axon terminals and, primarily, to a conduction block at the branching points where small axonal processes join the main neurite (Baylor and Nicholls, 1969; Jansen and Nicholls, 1973; Yau, 1976; Van Essen, 1973).

Existing electrophysiological research, analyzing the cellular mechanism of non-associative learning in the swimming (Brunelli *et al.* 1985*a*, 1986; Debski and Friesen, 1987; Nusbaum and Kristan, 1986) and shortening (Bagnoli *et al.* 1973; Belardetti *et al.* 1982; Frank *et al.* 1975) behaviour patterns, triggered by a light touch to the skin, has made it possible to demonstrate a clear effect of the endogenous monoamine serotonin (5-HT) (Lent and Frazer, 1977; Lent *et al.* 1979) on the AHP of T neurones. A previous paper (Belardetti *et al.* 1984) has shown that AHP amplitude can be depressed for prolonged periods either by the activation of serotonergic Retzius cells (a pair of giant neurones located in each segmental ganglion) or by 5-HT application. The effect is dose-dependent and is partially blocked by the 5-HT antagonist methysergide.

In this paper we have investigated the mechanism of action of 5-HT on the AHP of T sensory neurones. Since the AHP in these cells is partly due to the activation of the electrogenic Na^+/K^+-ATPase and partly to a Ca^{2+}-dependent K^+ conductance ($g_{K,Ca}$) (Baylor and Nicholls, 1969; Jansen and Nicholls, 1973; Van Essen, 1973), we performed two series of experiments: in the first, we tested the effect of 5-HT on the residual AHP after inhibiting $g_{K,Ca}$; in the second, we investigated the effect of 5-HT on T cells in which the activity of the Na^+/K^+-ATPase was modified.

The data indicate that 5-HT may act through the inhibition of the Na^+/K^+ electrogenic pump.

Materials and methods

Adult leeches of the species *Hirudo medicinalis* were purchased from a local supplier and kept in a well-aerated aquarium at 15°C. The animals were anaesthetized with 10% ethanol in water and pinned, ventral side up, to the paraffin wax floor of a plastic chamber. The ventral nerve cord was exposed by cutting the body wall along the midline and opening the ventral sinus. A short chain of ganglia was isolated at midbody level and pinned to the bottom of a small recording chamber coated with Sylgard (Dow Corning).

Unless otherwise stated, the following leech saline was used: 115 mmol l^{-1} NaCl, 4 mmol l^{-1} KCl, 1.8 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} glucose, buffered to pH 7.4 with Tris–maleate.

In some experiments trains of depolarizing pulses (200 ms, 2–3 s^{-1}, 25 s duration) were injected intracellularly into T cells; the discharge frequency of each series of trials was kept constant by adjusting the amount of current injected. Microelectrodes filled with 3–4 mol l^{-1} potassium acetate with resistances ranging from 40 to 100 MΩ were used for intracellular recordings and stimulations.

In one group of experiments the temperature was lowered by circulating a mixture of acetone and dry ice on the external surface of the recording chamber.

The temperature of the bath was constantly monitored with an electronic thermal probe. The shift produced by temperature variation was detected by a second microelectrode placed in the recording chamber and algebraically subtracted from the voltage measured by the microelectrode in the cell. The earth wire was located far from the recording chamber, using an agar/salt bridge. Serotonin (serotonin creatinine sulphate) (Sigma) was freshly dissolved in saline.

Only T cells with a resting potential of at least 40–50 mV, an input resistance greater than 20 MΩ and an action potential of 60–80 mV were selected. The AHP is very sensitive to the quality of the impalement: therefore, only cells with an AHP of at least 10–15 mV were used for the experiments.

During the recording session, the ganglia were perfused at a rate of 1.5 ml min^{-1} using a peristaltic pump. All the experiments were displayed on the screen of a storage oscilloscope and collected on a video recorder connected to a pulse code modulator (PCM 501 ES) (Sony).

Results

Effect of serotonin application

We measured the effects of different concentrations of 5-HT on some electrophysiological parameters of T sensory neurones. As pointed out in a previous paper (Belardetti *et al.* 1984), although a dose-dependent reduction of the AHP occurred, we have never seen consistent changes in the shape of the action potential (see Fig. 4A) or in the input resistance (see Fig. 1B), or a modification of the resting potential (see Fig. 1C). When 50 μmol l^{-1} 5-HT was applied, the maximal effect on AHP amplitude occurred 10 min after the removal of 5-HT by a saline wash; with 10 μmol l^{-1} 5-HT the maximal effect occurred 10 min after 5-HT application (Fig. 1).

Different approaches were used to investigate the mechanisms underlying 5-HT modulation of the AHP recorded in T neurones. Responses to 5-HT were first examined after the addition of $g_{K,Ca}$ blockers ($BaCl_2$ or $CdCl_2$), which eliminated the component that contributes to the generation of the AHP. The effects of 5-HT were then studied after increasing or decreasing the activity of the Na^+/K^+-ATPase.

Effect of serotonin after application of $BaCl_2$

In a preparation in which the AHP had been generated by trains of action potentials elicited by intracellular depolarizing pulses, $CaCl_2$ was completely replaced by $BaCl_2$ (1.8 mmol l^{-1}) to block $g_{K,Ca}$ from the intracellular side of the Ca^{2+} channel (Hagiwara, 1981; Meech, 1978; Paupardin-Tritsch *et al.* 1981). A 5 min application of $BaCl_2$ produced a reversible reduction of AHP amplitude to 60 % of the initial response (taken as 100 %). Prolonged applications (up to 30 min) did not reduce the AHP to a greater degree (Fig. 2B). During the 5 min $BaCl_2$ application the spike shape changed dramatically: the repolarization slowed and the undershoot disappeared (see Fig. 4B). After the initial 5 min of $BaCl_2$

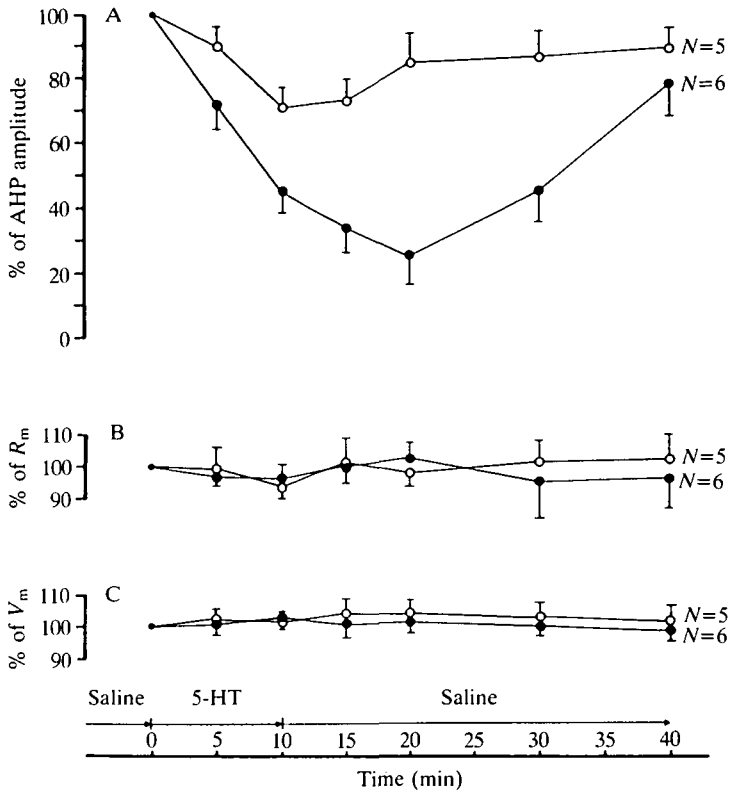

Fig. 1. (A) Trains of depolarizing pulses were injected intracellularly into T neurones. The preparation was perfused for 10 min with 50 μmol l^{-1} (●) or 10 μmol l^{-1} 5-HT (○). The after-hyperpolarization (AHP) amplitude of each cell (mean+S.E.M.) refers to the initial AHP amplitude, taken as 100% (also in Figs 2 and 3). A prolonged and dose-dependent reduction of the AHP amplitude was observed. (B) Input resistance (R_m) during the experiment illustrated in A. (C) Resting potential (V_m) during the experiment illustrated in A. *N* indicates the number of experiments performed (in all the figures).

application, perfusion with the blocking agent was continued and 50 or 10 μmol l^{-1} 5-HT was applied for 10 min. A further clear-cut reduction of AHP amplitude (to 25% of the initial response with 50 μmol l^{-1} 5-HT or 40% with 10 μmol l^{-1} 5-HT) was observed (Fig. 2), which agreed with the kinetics shown by 5-HT alone (Fig. 1A). The effect of the monoamine reversed after a 15 min wash in saline with $BaCl_2$, and an additional 15 min of perfusion with normal physiological solution reversed the effect of $BaCl_2$, restoring, almost completely, the AHP amplitude. The effects are illustrated and summarized in Fig. 2B.

Effect of serotonin after application of $CdCl_2$

A similar experiment was performed using 0.1 mmol l^{-1} $CdCl_2$ in normal saline

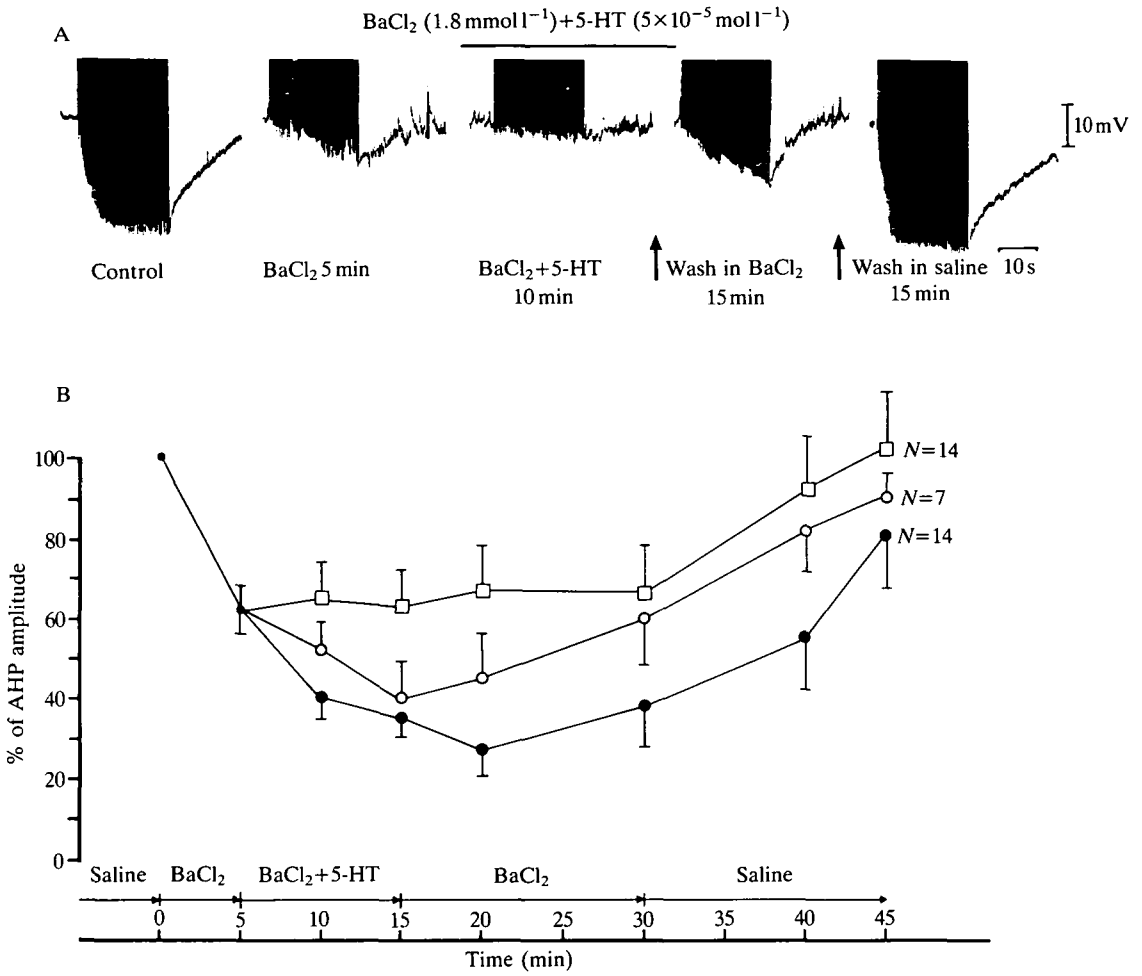

Fig. 2. (A) A train of depolarizing pulses was injected intracellularly into a T cell. The preparation was then perfused for 5 min with saline in which $CaCl_2$ was completely replaced with $BaCl_2$ (1.8 mmol l^{-1}); another depolarizing train was then delivered. A reduction of the AHP amplitude was observed. Perfusion with $BaCl_2$ was maintained for up to 30 min and 50 μmol l^{-1} 5-HT was applied for 10 min; we observed a further clear-cut reduction of the AHP. The effect of the monoamine reversed after 15 min of perfusion in saline with $BaCl_2$; an additional 15 min perfusion with normal physiological solution reversed the effect of $BaCl_2$. (B) Graph showing the mean+S.E.M. of the AHP amplitudes during (□) prolonged application of $BaCl_2$, (○) application of $BaCl_2$ and 10 μmol l^{-1} 5-HT, and (●) application of $BaCl_2$ and 50 μmol l^{-1} 5-HT.

to block $g_{K,Ca}$ from the extracellular side of the Ca^{2+} channel (Hagiwara, 1981; Madison and Nicoll, 1982; Meech, 1978) (Fig. 3). The same results were obtained as in the previous experiment. In this case the trace was less noisy with no spontaneous spikes after the testing train: this is probably attributable to the fact

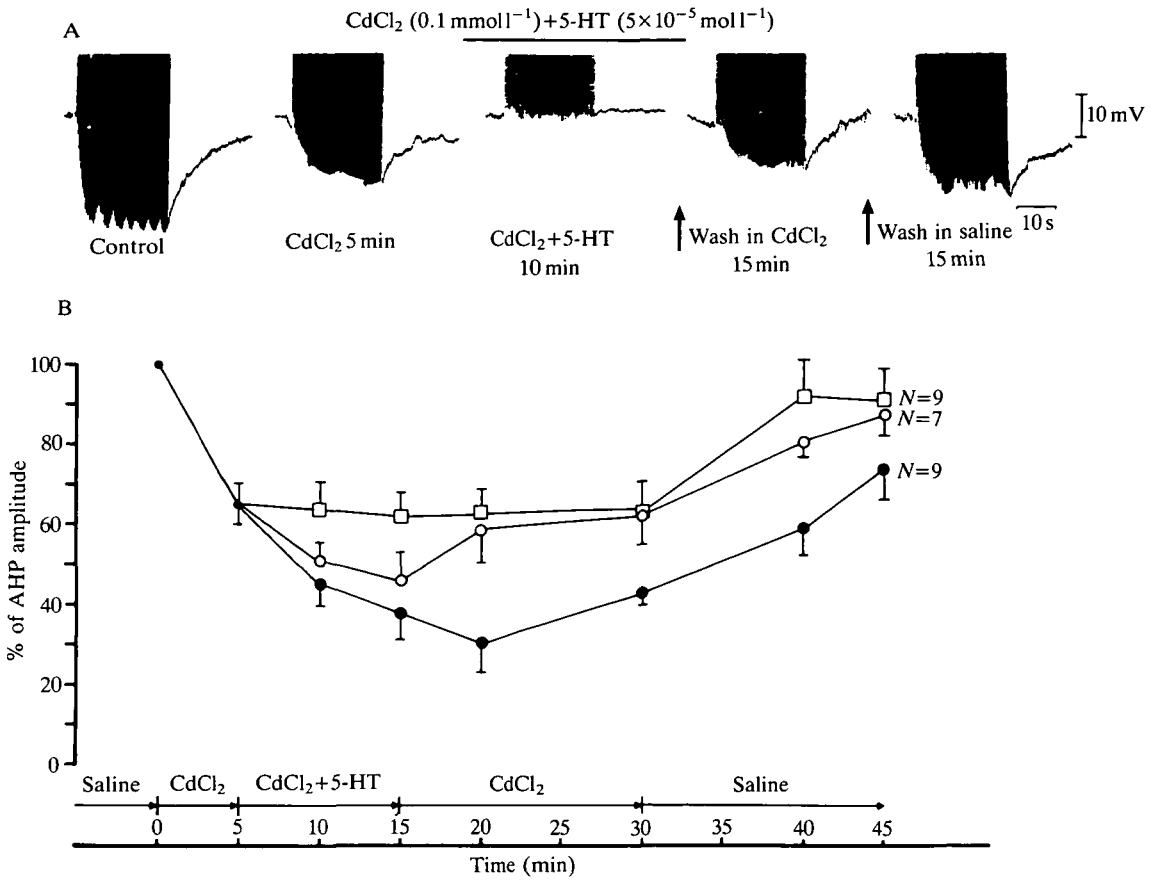

Fig. 3. (A) 0.1 mmol l^{-1} $CdCl_2$ in normal physiological solution was applied for 5 min, producing a reduction of AHP amplitude. Continued perfusion with this blocking agent plus 50 μmol l^{-1} 5-HT for 10 min gave a further reversible reduction in AHP amplitude. (B) Graph showing the mean+S.E.M. of the AHP amplitudes during: (□) prolonged application of $CdCl_2$, (○) application of $CdCl_2$ and 10 μmol l^{-1} 5-HT, and (●) application of $CdCl_2$ and 50 μmol l^{-1} 5-HT.

that, in the experiment with $BaCl_2$, Ca^{2+}-free solution was used and consequently cellular excitability increased. A 5 min application of $CdCl_2$ induced a decrease of AHP amplitude to 60% of the initial response, with no further reduction when application was continued for up to 30 min (Fig. 3B). A 5 min application of $CdCl_2$ caused an increase in spike duration and a disappearance of the undershoot (Fig. 4C). A 10 min incubation with 5-HT led to a further depression of the AHP amplitude, to approximately 30% of the initial response with 50 μmol l^{-1} 5-HT or approximately 45% with 10 μmol l^{-1} 5-HT (Fig. 3). The effect was partially restored after washing out the 5-HT (Fig. 3B).

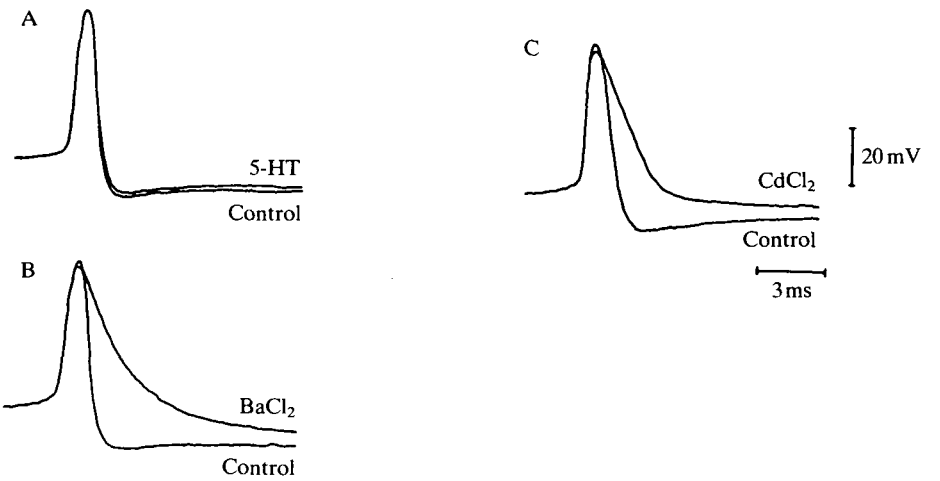

Fig. 4. (A) Action potential shape before and after 10 min of perfusion with 50 μmol l^{-1} 5-HT. (B) Changes in the action potential shape after 5 min of $BaCl_2$ application. (C) Changes in the action potential shape after 5 min of $CdCl_2$ application.

Effect of serotonin after injection of Na^+

When T neurones were impaled with a 3 mol l^{-1} sodium acetate microelectrode a clear hyperpolarization (about 15 %) could be observed (Fig. 5A). It is assumed that this effect is due to the activation of the Na^+/K^+ electrogenic pump following Na^+ leakage from the microelectrode into the cell (Jansen and Nicholls, 1973). When potassium acetate microelectrodes were used, no variation of membrane voltage (V_m) could be detected (Fig. 5B).

In some experiments we perfused the ganglia with 50 μmol l^{-1} 5-HT for 10 min before penetrating T cells with sodium acetate electrodes and we obtained a depolarization of membrane voltage of about 15 % (Fig. 5C). This effect may be explained by an inhibitory action of 5-HT on the Na^+/K^+-ATPase so that Na^+ leaking into T cells cannot be pumped out and a depolarization results. Fig. 5D clearly illustrates the comparative membrane voltages of T neurones after penetration with 3 mol l^{-1} potassium acetate or 3 mol l^{-1} sodium acetate with or without the application of 5-HT.

Effect of serotonin after application of CsCl

In experimental preparations in which the ganglia were perfused with K^+-free saline, we obtained a biphasic variation of membrane voltage. An early hyperpolarization phase, probably due to a greater K^+ outflow caused by the rise of the chemical gradient, was followed by a delayed depolarization phase which was sensitive to ouabain and, therefore, mainly due to the inactivation of the electrogenic pump, which is known to be sensitive to the external K^+ concentration (Fig. 6A). These results agree with those found in P and N cells by Schlue and Deitmer (1984).

If, at the end of the depolarization phase, we perfused the ganglia with K^+-free

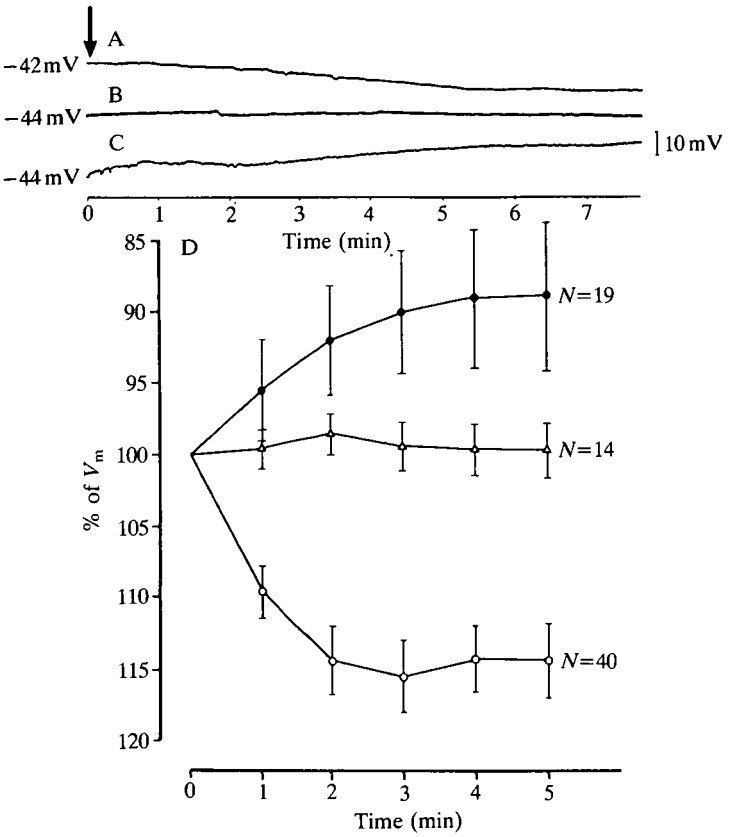

Fig. 5. (A) Membrane potential (V_m) of a T neurone after penetration with a 3 mol l^{-1} sodium acetate microelectrode. A hyperpolarization due to the activation of the electrogenic pump can be observed. (B) V_m of a T neurone after penetration with a 3 mol l^{-1} potassium acetate microelectrode. No variations of V_m were observed. (C) V_m of a T neurone after penetration with a 3 mol l^{-1} sodium acetate microelectrode in a preparation perfused for 10 min with 50 μmol l^{-1} 5-HT before penetration. A clear-cut depolarization from the initial V_m was noted. (D) Mean±S.E.M. of V_m: (○) 3 mol l^{-1} sodium acetate microelectrode; (△) 3 mol l^{-1} potassium acetate microelectrode; (●) 3 mol l^{-1} sodium acetate microelectrode plus a 10 min exposure to 50 μmol l^{-1} 5-HT. In all the cases V_m values of each cell are referred to the initial resting potential, taken as 100%. This also applies to Figs 6 and 7.

saline containing 4 mmol l^{-1} Cs^+, a Na^+/K^+-ATPase activator (Skou, 1960, 1965), we observed a membrane repolarization (Fig. 6C). Alternatively, if 50 μmol l^{-1} 5-HT was applied 10 min before CsCl, the repolarization was greatly reduced (by approximately 30%) and delayed (Fig. 6B).

Effect of serotonin after cooling the ganglia

The V_m of T neurones was monitored during the reversible cooling of the preparation from 18 to 10°C. During cooling a depolarization of about 15% was

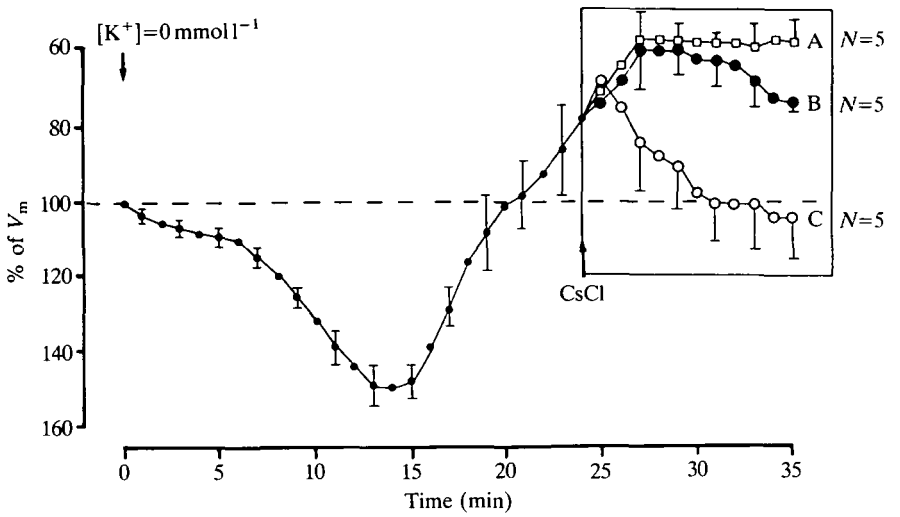

Fig. 6. Mean±S.E.M. of V_m for T neurones (N=5) perfused with K^+-free saline for about 25 min (NaCl was raised to 119 mmol l^{-1} to maintain iso-osmolarity). Two phases are evident: a hyperpolarization followed by a depolarization. (□) Prolonged application of K^+-free solution (A). (○) After depolarization, five cells were perfused with saline in which 4 mmol l^{-1} KCl was completely replaced with 4 mmol l^{-1} CsCl. A repolarization occurred (C). (●) After depolarization, five cells were treated for 10 min with 50 μmol l^{-1} 5-HT and then perfused with saline containing CsCl. Repolarization was reduced (B).

obtained (Fig. 7). When the temperature was restored to 18°C a repolarization was observed; this was sensitive to ouabain and strophantidin and often rose to 105–110 % of the initial V_m. This effect is due to the inhibition and reactivation of the Na^+/K^+-ATPase (Baylor and Nicholls, 1969; Kuba and Koketsu, 1979).

The same procedure was adopted after a 10 min application of 50 μmol l^{-1} 5-HT, producing a larger depolarization phase of about 25–30 % and a smaller repolarization phase reaching 90 % of the initial voltage (Fig. 7).

The effect of serotonin after ouabain treatment

The Na^+/K^+-ATPase was blocked with 2×10^{-4} mol l^{-1} ouabain or strophantidin in order to study the effect of 5-HT on the residual AHP; however, the remaining AHP was often very small (Baylor and Nicholls, 1969; Jansen and Nicholls, 1973) and it was difficult to maintain a constant discharge frequency, probably because of an excess of Na^+ in the T cell that cannot be pumped out. In a few cases in which a residual AHP could be detected, 5-HT was unable to provoke a further reduction of the AHP amplitude (data not shown).

Discussion

In a previous paper (Belardetti *et al.* 1984) it was shown that 5-HT perfusion or

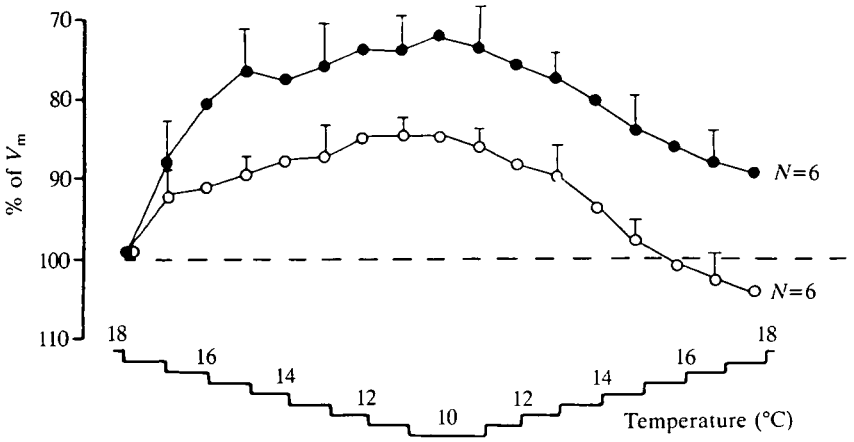

Fig. 7. (○) Mean+S.E.M. of V_m in T neurones after cooling to 10°C and rewarming to room temperature (18°C). During cooling a depolarization took place, whereas during rewarming repolarization was observed. This is due to the inhibition and reactivation of the electrogenic pump. (●) 10 min before cooling, the ganglia were perfused with 50 μmol l^{-1} 5-HT. The application lasted 10 min after the cooling process had started. This produced a greater depolarization phase and a slower and smaller repolarization one. The entire cooling and rewarming procedure lasted 15–20 min.

the electrical stimulation of the serotonergic Retzius cells produced a long-lasting clear-cut reduction of AHP amplitude in T sensory neurones. This effect depended on the concentration of the monoamine, was inhibited by the antagonist methysergide and was not blocked by bathing the ganglion with high-Mg^{2+} saline. In agreement with the observations of Belardetti *et al.* (1984), we have never seen consistent changes of T cell resting potential, input resistance or action potential shape with 5-HT perfusion (Figs 1, 4A).

From these preliminary observations, it was difficult to ascribe the mechanism of action of serotonin to an inhibition of $g_{K,Ca}$, which is known to make a partial contribution to the AHP (Baylor and Nicholls, 1969; Jansen and Nicholls, 1973); in fact, no increase of input resistance was observed. A simple short-circuit of the currents generating the AHP probably also fails to explain the action of 5-HT; a 70–75% decrease of the AHP amplitude, obtained with 50 μmol l^{-1} 5-HT perfusion for 10 min, should result from an identical reduction of the input resistance. A possible shunting action of 5-HT is also unlikely because it would affect the AHPs identically, irrespective of their amplitudes, whereas large AHPs are more depressed than the small ones (Belardetti *et al.* 1984).

To clarify the mechanism of action of 5-HT on T neurones, we performed two series of experiments. In the first series, we blocked $g_{K,Ca}$ with $BaCl_2$ or $CdCl_2$ and demonstrated that 5-HT was still able to reduce AHP, even after $g_{K,Ca}$ inhibition. We also observed that Ba^{2+} or Cd^{2+} application caused an unexpectedly large reduction (about 40%) of the AHP amplitude, which is known to depend greatly

on the activation of the electrogenic pump in T neurones. This may be caused by the reduction of input resistance that accompanies $BaCl_2$ or $CdCl_2$ application (about 16% and 11%, respectively), probably due to an increase of membrane permeability following variation of Ca^{2+} concentration. Moreover, an interaction between $g_{K,Ca}$ and electrogenic pump activity cannot be ruled out. An involvement of 5-HT in $g_{K,Ca}$ is made unlikely by the finding that, in experiments in which it was possible to get a residual AHP following ouabain or strophantidin treatment, 5-HT was ineffective in provoking a further reduction of the AHP amplitude.

We also tried to study the effect of 5-HT by holding T cells at a membrane potential close to the K^+ reversal potential, but the long projections of the neurite do not allow good space-clamping even with double-electrode voltage-clamp techniques (Jansen and Nicholls, 1973; Stewart *et al.* 1989).

In the second series of experiments, it has been possible to collect evidence for a direct inhibition of the Na^+/K^+-ATPase by 5-HT. Injection of Na^+ into T neurones after perfusion with 5-HT led to a depolarization instead of the normal, ouabain-sensitive hyperpolarization. This effect may be explained by an inhibition of the electrogenic pump by 5-HT and by leakage of positive ions into the cell. These ions cannot then be pumped out.

Moreover, 5-HT caused a reduction of the repolarization normally obtained with the Na^+/K^+ pump activator Cs^+ after K^+-free perfusion. Cs^+ has a permeability of less than 8% for the K^+ channel (taking the permeability of K^+ for its channel as 100%) (Stryer, 1982) and, therefore, its effect on the Na^+/K^+ electrogenic pump is not masked by the current through the K^+ channel, as happens when K^+ itself is used to reactivate the pump.

In addition, 5-HT increased the depolarization phase during cooling of the ganglia and produced a considerable reduction in speed of repolarization when the temperature was returned to 18°C and, consequently, the electrogenic pump should have been reactivated. The inhibitory action of 5-HT on the electrogenic pump is a surprising result since, although examples of positive modulation of the Na^+/K^+-ATPase caused by neurotransmitters have been described (Kuba and Koketsu, 1979; Phillis and Wu, 1981; Thomas, 1972), inhibitory effects have rarely been found and have been investigated only with biochemical methods (Lingham and Sen, 1983; Mourek, 1988).

A further observation is that 5-HT has a reduced effect on the AHP amplitude of P sensory cells and no effect at all on that of N neurones (Brunelli *et al.* 1985*b*), the AHP of which is more dependent on $g_{K,Ca}$ (Baylor and Nicholls, 1969; Jansen and Nicholls, 1973; Van Essen, 1973).

A possible effect of 5-HT on the Na^+/K^+-ATPase might be to change the transport coupling ratio, making the pump work with a less electrogenic mechanism. Through the inhibition of the electrogenic pump, 5-HT may have a role in removing the conduction blocks caused by the AHP. This potentiating effect may, at least in part, explain the behavioural sensitization and dishabituation induced by 5-HT and observed in experiments on the induction of swimming

(Brunelli *et al.* 1985*a*, 1986; Debski and Friesen, 1987; Nusbaum and Kristan, 1986) and on the 'fast conducting system' (Belardetti *et al.* 1982; Frank *et al.* 1975).

It will be interesting to investigate whether a similar inhibitory action of the electrogenic pump can be found in other leech neurones, in other nervous systems or in non-excitable tissues.

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