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MODULATION OF IONIC CURRENTS BY SYNAPTIC ACTION AND 5-HT APPLICATION IN THE IDENTIFIED HEART EXCITATORY NEURONE OF THE AFRICAN GIANT SNAIL, *ACHATINA FULICA* FÉRUSSAC

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Summary

In the African giant snail, *Achatina fulica* Férussac, the ionic mechanisms underlying slow depolarization of a heart excitatory neurone, PON, induced by the activity of two cerebral ganglionic neurones, d-RCDN and d-LCDN, were investigated under voltage-clamp.

The slow depolarization of PON that was induced by the activity of the cerebral neurones was blocked by the serotonin (5-HT) antagonist, methysergide. Bath application of 5-HT to the axotomized PON produced a similar slow depolarization, which was also blocked by methysergide. These results suggest that the neurotransmitter of d-RCDN and d-LCDN is 5-HT.

Under voltage-clamp, activity of the cerebral neurones usually produced an inward shift in the holding current of PON with a decrease of conductance. Ionic substitution experiments and injection of Cs^+ into PON showed that the response was mainly due to a decrease in K^+ conductance. In some cases, this inward shift showed two components: an early component with increased conductance and a late one with decreased conductance. The early component was not decreased by Cs^+ injection but was augmented by EGTA injection into PON, which may suggest the involvement of a Ca^{2+} conductance in this synaptic response.

Application of 5-HT produced a similar inward shift in holding current which was also mainly the result of a decrease in the background K^+ current. 5-HT was also found to increase the voltage-dependent Ca^{2+} current and the inward rectifying K^+ current.

The significance of these results is discussed in relation to the heart regulation of this snail.

Introduction

In addition to the classical 'fast' synaptic transmission which leads to the opening of previously closed ion channels, 'slow' synaptic transmission, which

Key words: snail neurone, modulation of ionic current, serotonin, voltage-clamp.

often includes the modulation of already functioning ion channels, has been found in both vertebrate and invertebrate tissues (Kehoe & Marty, 1980; Hartzell, 1981). Potassium channels are well-known targets of such modulation. For example, in the sensory neurone of *Aplysia*, a serotonin (5-HT)-sensitive K^+ channel (S channel), which is open at the resting potential, is closed by 5-HT or the small cardioactive peptides A and B (SCPs), probably mediated by cyclic AMP-dependent protein phosphorylation (Klein & Kandel, 1980; Klein, Camardo & Kandel, 1982; Siegelbaum, Camardo & Kandel, 1982; Abrams *et al.* 1984). The opening probability of this channel is increased by a molluscan cardioactive peptide, FMRFamide (Belardetti, Kandel & Siegelbaum, 1986). Neurotransmitter-induced closure of this channel is considered to be the mechanism underlying sensitization, a model for short-term memory (Kandel & Schwartz, 1982). A similar 5-HT-sensitive K^+ channel has also been reported in *Helix* and *Hermissenda* neurones (Paupardin-Tritsch, Deterre & Gerschenfeld, 1981; Jacklet & Acosta-Urquidi, 1985). Modulation of K^+ channels by neurotransmitters is also known in some vertebrate and invertebrate neurones (Paupardin-Tritsch, Colombaioni, Deterre & Gerschenfeld, 1985; Benson & Levitan, 1983; Cottrell, Davies & Green, 1984; Brown & Adams, 1980; Akasu, Nishimura & Koketsu, 1983).

Further targets for such modulation are Ca^{2+} channels, and a well-known example is the Ca^{2+} channel of vertebrate heart muscle, where β-adrenergic agents increase Ca^{2+} current, and this is also considered to be mediated by cyclic AMP-dependent protein phosphorylation (Osterrieder *et al.* 1982; Cachelin, De Peyer, Kokubun & Reuter, 1983). In some *Helix* neurones, Ca^{2+} current is increased by 5-HT (Paupardin-Tritsch, Hammond & Gerschenfeld, 1986*a*), and in other cells it is decreased by FMRFamide (Colombaioni, Paupardin-Tritsch, Vidal & Gerschenfeld, 1985). 5-HT-induced increase of the Ca^{2+} current of the *Helix* neurone is probably mediated by cyclic GMP-dependent protein kinase (Paupardin-Tritsch *et al.* 1986*b*). A similar 5-HT-induced increase of Ca^{2+} current has been reported in *Hermissenda* neurones (Jacklet & Acosta-Urquidi, 1985). In chick dorsal root ganglion cells, γ-aminobutyric acid, dopamine and noradrenaline decrease Ca^{2+} current (Deisz & Lux, 1985; Marchetti, Carbone & Lux, 1986).

In the central nervous system of the African giant snail, *Achatina fulica* Férussac, several heart regulatory neurones have been identified and their interconnections have been described (Furukawa & Kobayashi, 1978*a*,*b*). Among them, two cerebral ganglion cells, the dorsal right cerebral distinct neurone (d-RCDN) and the dorsal left cerebral distinct neurone (d-LCDN), produce a slow depolarization in the periodically oscillating neurone (PON) which is the most effective heart excitor.

In the present study, the ionic mechanisms underlying this slow depolarization, and a similar depolarization produced by application of 5-HT, were examined under voltage-clamp.

Table 1. *Composition of experimental solutions (mmol l^{-1})*

Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	$BaCl_2$	TrisCl	TEACl	$CoCl_2$	Glu	Hepes
NPS	61	3·3	10·7	13					5	10
3K	54·4	9·9	10·7	13					5	10
Na^+-, K^+-, Ca^{2+}-free				13		64·3		10·7	5	10
Na^+-, Ca^{2+}-free, 10K		33		13		31·3		10·7	5	10
Ca^{2+}-free	61	3·3		13				10·7	5	10
*TEA^+, Ba^{2+}		3·3		13	10·7		61		5	10

NPS, normal physiological solution.
Glu, glucose.
* 5 mmol l^{-1} 4-AP (4-aminopyridine) was also added in some cases.
pH adjusted to 7·5 by titration with HCl or NaOH.

Materials and methods

The African giant snail, *Achatina fulica* Férussac, captured in Okinawa and transported by air to Hiroshima, was bred in our laboratory at 24 °C. Circumoesophageal ganglia were dissected out of the animal. The connective capsule and the inner sheath covering the dorsal surface of the right parietal ganglion and cerebral ganglia were completely removed by dissection to expose the nerve cells. The preparation was pinned to the bottom of an experimental chamber coated with silicone resin and was continuously perfused with normal physiological solution. The effective volume of the chamber was 0·5 ml and the perfusion rate was 2–5 ml min^{-1}. The temperature of the perfusate was maintained at 24 °C by a thermoelectric device. The ionic compositions of experimental solutions are listed in Table 1.

Intracellular recordings were made from cerebral neurones (d-RCDN or d-LCDN) using glass microelectrodes filled with a mixture of 3 mol l^{-1} potassium acetate and 0·1 mol l^{-1} KCl (resistance about 5 MΩ). Electrical stimulation was carried out through the recording electrode. The neurone was driven to burst by a depolarizing current pulse, and the number of spikes in the burst was not strictly controlled. However, a given duration of depolarizing pulse evoked a fairly constant number of spikes and produced reproducible synaptic responses in PON. PON was voltage-clamped by a two-microelectrode method as described previously (Furukawa & Kobayashi, 1986) with modifications as follows. Both recording and current-passing electrodes were silver painted to within 2 mm of the tip, insulated with nail polish and filled with the above-mentioned mixture. The resistance of the voltage-recording electrode was about 5 MΩ and that of the current-passing electrode was 2–3 MΩ. The silver screen of each electrode was driven by positive feedback from a unity gain in the head stage, which reduced the stray capacitance of the electrodes and improved the frequency response of the system. A grounded shield was placed between the two electrodes to minimize coupling between them. Under these conditions, the rise time of a square voltage pulse was less than 200 μs. Membrane currents were read as a voltage drop across a

1-MΩ resistor interposed in the feedback loop or were measured by a virtual ground circuit. Series resistance compensation was carried out by subtracting the appropriate fraction of the current signal from the summing point of the voltage-clamp circuit to give the fastest capacitive transient.

In some experiments, Cs^+ or EGTA was injected into PON ionophoretically by using a separate electrode containing $2\,mol\,l^{-1}$ CsCl or $0{\cdot}5\,mol\,l^{-1}$ EGTA. The injection was carried out under voltage-clamped conditions at an intensity of $0{\cdot}5\,\mu A$ for 10 min.

In a few experiments, stimulation of the branch of the intestinal nerve arising from the suboesophageal ganglia, or extracellular recording from this nerve, was performed as described previously (Furukawa & Kobayashi, 1987*a*).

The data were displayed on an oscilloscope (Nihon Kohden, VC-10) and stored on an FM tape recorder (Sony, A-85) for later analysis. Permanent records were produced upon a pen-recorder (Nihon Kohden, PMP-3000) or an x–y recorder (Yokogawa, type 3077). For the subtraction of capacitive and linear leak currents, currents elicited by identical hyperpolarizing and depolarizing command pulses were summed by a signal averager (Nihon Kohden, DAT-1100).

For some experiments, PON was axotomized by cutting the septum between the visceral and the right parietal ganglion. No synaptic input was seen after this operation. The axotomized cell was kept in the perfused normal physiological solution for more than 30 min before the experiment, and was used only if the resting potential was greater than −40 mV and if an all-or-none action potential was given when depolarizing current was injected.

5-Hydroxytryptamine creatinine sulphate (5-HT, Sigma) was dissolved in the experimental solution and applied by bath perfusion. Duration of the application was usually less than 5 min and an interval of at least 20 min was allowed between the 5-HT applications for reproducible responses. The 5-HT antagonist, methysergide-hydrogenmaleinate (methysergide, Sandoz) was also applied by bath perfusion 5–10 min before either stimulation of the cerebral cell or 5-HT application. These drugs were freshly dissolved before experiments as $1\,mmol\,l^{-1}$ stock solutions, and stored in the refrigerator for later use.

Results

Cerebral neurones or 5-HT induce slow depolarization in PON

The activity of two cerebral neurones, d-RCDN and d-LCDN, produces a slow depolarizing response in PON (Furukawa & Kobayashi, 1987*b*). This slow depolarization was found to be depressed reversibly by the 5-HT antagonist, methysergide ($50\,\mu mol\,l^{-1}$, Fig. 1A), in six preparations. Similarly, 5-HT produced a slow depolarization in axotomized PON, which was also depressed by methysergide (Fig. 1B) in four preparations. 5-HT appeared to be acting directly upon PON since the neurone was isolated by axotomy, and the soma of molluscan neurones is known to lack synaptic contacts. Higher concentrations of methysergide produced a complete block which was not reversible over the experimental

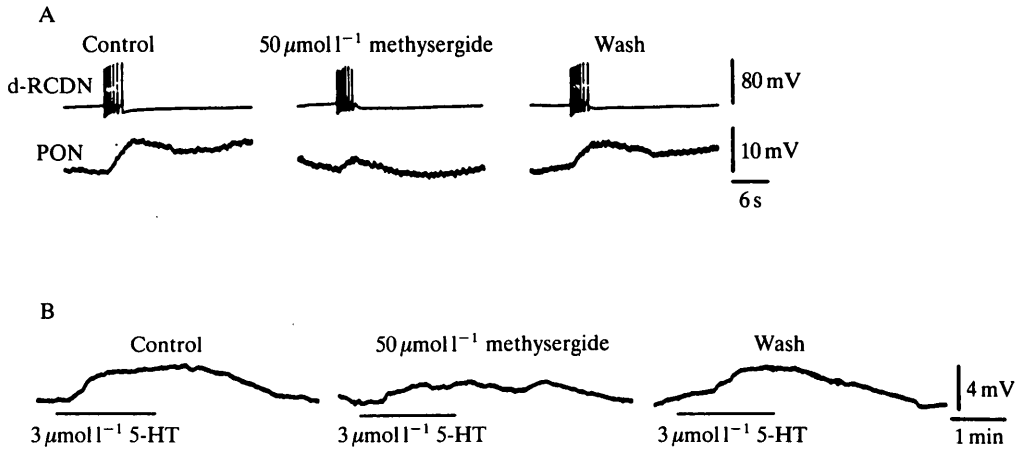

Fig. 1. (A) Blocking action of methysergide on the slow depolarization of PON induced by activity of d-RCDN. PON was hyperpolarized to −80 mV. d-RCDN was made to fire by a depolarizing current injection. Spike number was 8 in the control trace, and 9 in the 50 μmol l^{-1} methysergide and Wash traces. (B) Blocking action of methysergide on the slow depolarization of axotomized PON induced by 5-HT. Membrane potential of PON was −40 mV.

time course (less than 2 h). These results suggest that 5-HT is the neurotransmitter of the two cerebral neurones. To examine this hypothesis, the ionic mechanism of the synaptic action was compared with that of the 5-HT action.

Ionic mechanisms of the slow depolarization of PON by cerebral neurones

When the membrane potential of PON was held at −50 mV, activity in the cerebral neurone induced an inward shift in the holding current with a decrease of conductance (Fig. 2Ai). At a more negative holding potential, less current shift was produced (Fig. 2Aii). After the burst in the cerebral neurone, there was a slow recovery of the current level (Fig. 2A). Similar results were obtained in 10 preparations out of 12. In the other two preparations a change of conductance was not observed.

To examine whether the decrease in conductance included a decrease in K^+ conductance, PON was held at −50 mV and the effects of a d-LCDN burst upon conductance observed in normal physiological solution were compared with the effects obtained at three times the K^+ concentration, in 3K solution (see Table 1). The inward shift in holding current produced by the d-LCDN burst was larger in the normal solution (Fig. 2Bi) than in the 3K solution (Fig. 2Ci). The current–voltage (I–V) relationships in normal solution, measured using 200-ms hyperpolarizing pulses, showed that the extra membrane current induced by the activity of d-LCDN (the difference between the open and closed circles in Fig. 2Bii) was reduced by hyperpolarization but not reversed. In 3K solution, the effect of d-LCDN activity upon the I–V curve was much smaller and showed a null effect at

−90 mV (Fig. 2Cii). Similar results were obtained in all tested preparations ($N = 5$). These results indicate that a decrease of K^+ conductance is involved in the slow depolarization induced by the cerebral neurones. However, since the null

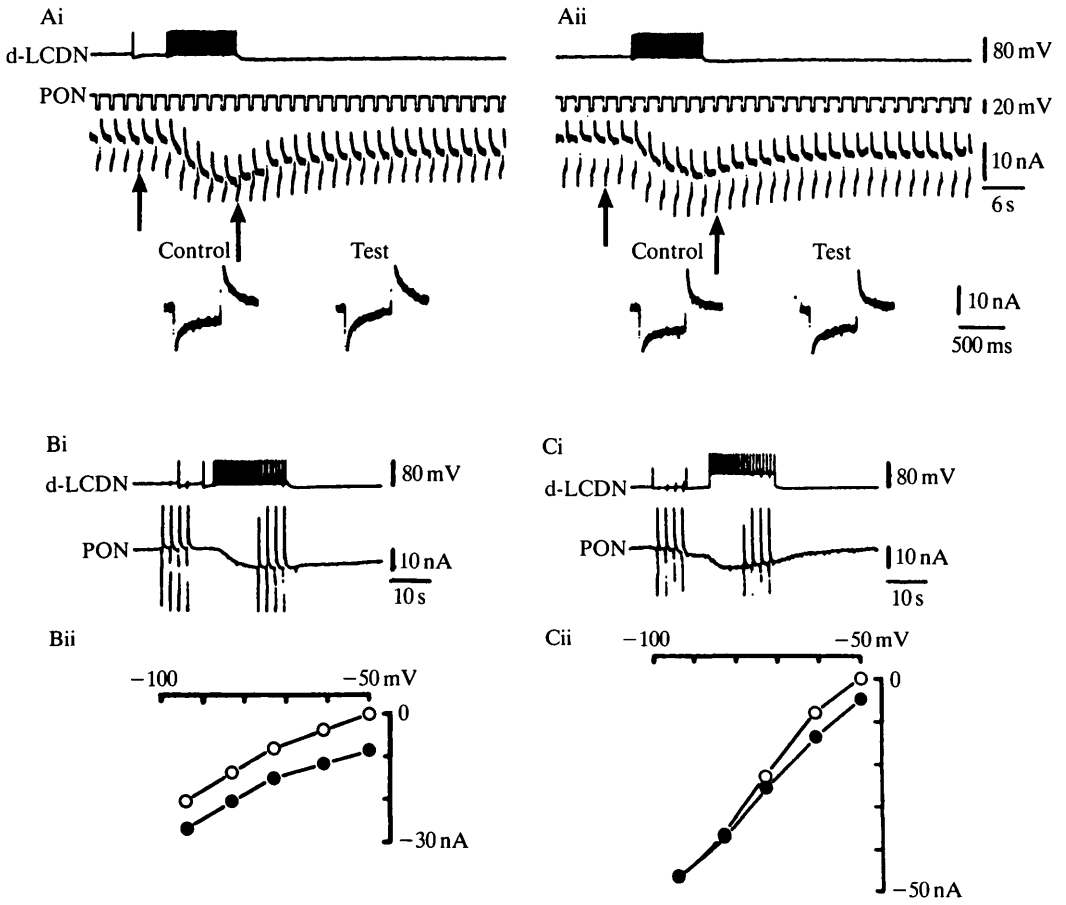

Fig. 2. (A) Inward shift in holding current of PON induced by activity of d-LCDN. Holding potential of PON was −50 mV in Ai and −70 mV in Aii. Hyperpolarizing command pulses (20 mV, 500 ms duration) were applied at 0·5 Hz to monitor the change of conductance. Arrows indicate selected currents which are displayed in lower insets at expanded time scale. Note the decrease of conductance in PON produced by activity of d-LCDN. Spike number was 42 in Ai and 39 in Aii. Ai and Aii were obtained from the same preparation. (B) I–V relationships of PON before and during a burst of d-LCDN in the normal physiological solution. (C) I–V relationships of PON before and during a burst of d-LCDN in 3K solution. Holding potential was −50 mV in both B and C. I–V relationships were measured by applying command pulses (200 ms duration), before and during a burst of d-LCDN, which are seen as vertical deflections in Bi and Ci. The amplitude of the current at the end of the pulse is plotted against the command voltage in Bii and Cii. The holding current before the burst of d-LCDN is drawn at 0 nA. Open circles, before the burst, closed circles, during the burst. d-LCDN was made to fire by a depolarizing current injection. Spike number was 35 in B and 31 in C. B and C were from the same preparation.

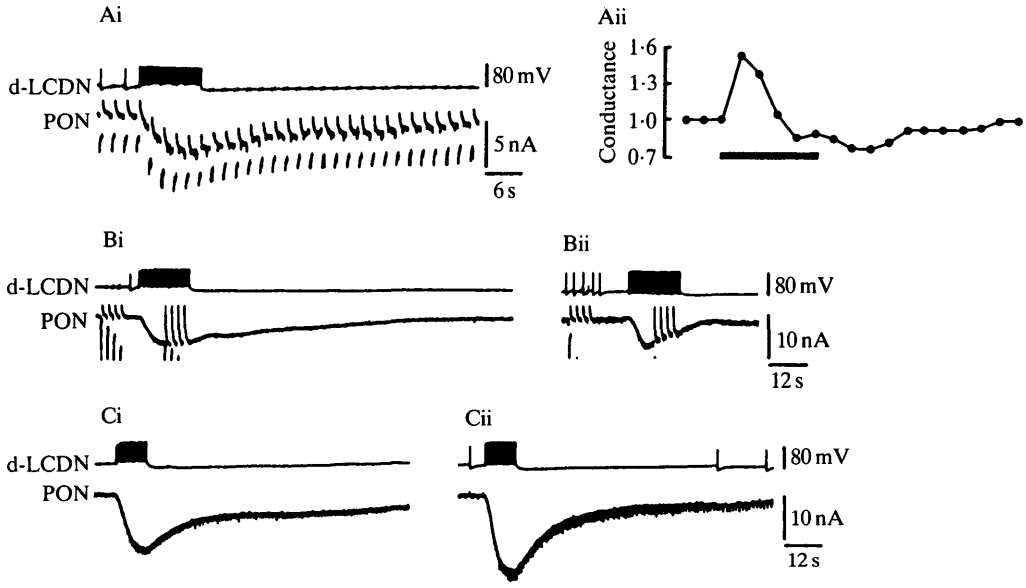

Fig. 3. (Ai) Inward shift in holding current of PON induced by activity of d-LCDN, preceded by a transient increase in conductance. Holding potential was −60 mV, and hyperpolarizing command pulses (20 mV, 500 ms duration) were applied at 0·5 Hz. d-LCDN was made to fire by depolarizing current injection. Spike number was 50. (Aii) Change in conductance of PON produced by a burst of d-LCDN, obtained from the same record as that displayed in Ai. Vertical scale indicates the conductance and control conductance is denoted as 1·0. Horizontal interval between points is 2 s. Bar indicates the duration of a burst of d-LCDN. (B) Effect of Cs^+ injection into PON on the inward shift in holding current induced by activity of d-LCDN. Holding potential was −60 mV. (Bi) Control; (Bii) after Cs^+ injection. d-LCDN was made to fire by depolarizing current injection. Spike number was 61 in Bi and 68 in Bii. Vertical deflections in the current traces are currents in response to the command pulses. (C) Effect of EGTA injection into PON on the inward shift in holding current induced by activity of d-LCDN. Holding potential was −50 mV. (Ci) Control; (Cii) after EGTA injection. d-LCDN was made to fire by a depolarizing current injection. Spike number was 37 in Ci and 40 in Cii.

potential in 3K solution was too negative for E_K, the involvement of other ionic mechanisms is also suggested.

In some preparations ($N = 3$), the activity in the cerebral cells produced a transient increase of conductance before the decrease in conductance (Fig. 3Ai). This can clearly be seen when conductance is plotted as a proportion of the value before the d-LCDN burst (Fig. 3Aii). Thus, activity of the cerebral cells induces a further change in PON, besides a decrease in K^+ conductance. To investigate further whether the inward shift in holding current induced by cerebral cell activity was dependent on K^+ conductance, Cs^+ was injected into PON, since this ion blocks K^+ channels (Colmers, Lewis & Wilson, 1982). Before Cs^+ injection, PON showed a steady inward shift in holding current during activity in d-LCDN and

quite a slow recovery after the burst (Fig. 3Bi). After Cs^+ injection, the inward shift began to decay during the d-LCDN burst, and there was rapid recovery (Fig. 3Bii). Although this result is consistent with the notion that the slow depolarization induced by the cerebral cells is due to a decrease in K^+ conductance, the peak level of the synaptic response did not change (Fig. 3Bii). This result, together with the transient increase in conductance (Fig. 3A), suggests that the slow depolarization of PON induced by the cerebral cells is due to two different conductance mechanisms: a decrease of K^+ conductance which persists after activity in the cerebral cells, and a transient increase in conductance of an unknown ion. As Mg^{2+} can be neglected, possible ions are Na^+, Ca^{2+} and Cl^-. Na^+ can be excluded as there was no effect on this response when half the Na^+ was replaced by $Tris^+$. Cl^- cannot be involved because Cl^- injection into PON had no effect. Thus, the only plausible mechanism appears to be an increase of Ca^{2+} conductance.

The possible involvement of Ca^{2+} could not be investigated by ionic substitution since this would modify the transmitter release. Instead, we investigated the effect of EGTA injection into PON upon the synaptic response, because the Ca^{2+} channel is known to be inactivated by an increase in $[Ca^{2+}]_i$ and that type of inactivation can be depressed by the injection of EGTA (Plant, Standen & Ward, 1983). The peak amplitude of synaptic response in PON produced by a burst of d-LCDN was clearly increased by EGTA injection into PON (Fig. 3C), consistent with the notion that an increase of Ca^{2+} current, in addition to a decrease of K^+ current, is concerned in the slow depolarization of PON induced by the cerebral cell activity.

Ionic mechanisms of the slow depolarization of PON by 5-HT

With the membrane potential of PON clamped at −50 mV, application of 5-HT, at concentrations above about $1\,\mu mol\,l^{-1}$, produced an inward shift in holding current with a decrease in conductance. Preliminary experiments showed that the response was unaffected by a decrease in Na^+ concentration, but was reduced by an increase in K^+ concentration.

The effect of K^+ concentration upon the 5-HT response was investigated in the absence of Na^+ and Ca^{2+}, and in the presence of Co^{2+}. The solutions were made by mixing the Na^+-, K^+-, Ca^{2+}-free solution and the Na^+-, Ca^{2+}-free, 10K solution (see Table 1). Quasi-steady-state I–V relationships were measured using 300-ms command pulses. Sodium-free conditions were employed because it was easier to measure the 5-HT-sensitive current in the absence of the Na^+ current. The measurements were made in the absence of Ca^{2+}, and in the presence of Co^{2+}, because 5-HT was found to increase the voltage-dependent Ca^{2+} current (as shown later).

5-HT produced an inward shift in holding current of PON (see upper inset of Fig. 4Ai) and decreased outward current at every tested voltage at a potassium concentration of $3{\cdot}3\,mmol\,l^{-1}$ (Fig. 4Ai). This 5-HT-sensitive current (i.e. difference current) showed little time dependency (see lower inset of Fig. 4Ai). The

5-HT-sensitive current became smaller with hyperpolarization but was not reversed (Fig. 4Aii; Fig. 5, circles). At a potassium concentration of 33 mmol l^{-1}, 5-HT produced an outward shift of holding current (see upper inset of Fig. 4Bi). The 5-HT-sensitive current reversed at about −40 mV (Fig. 4Bii; Fig. 5, squares).

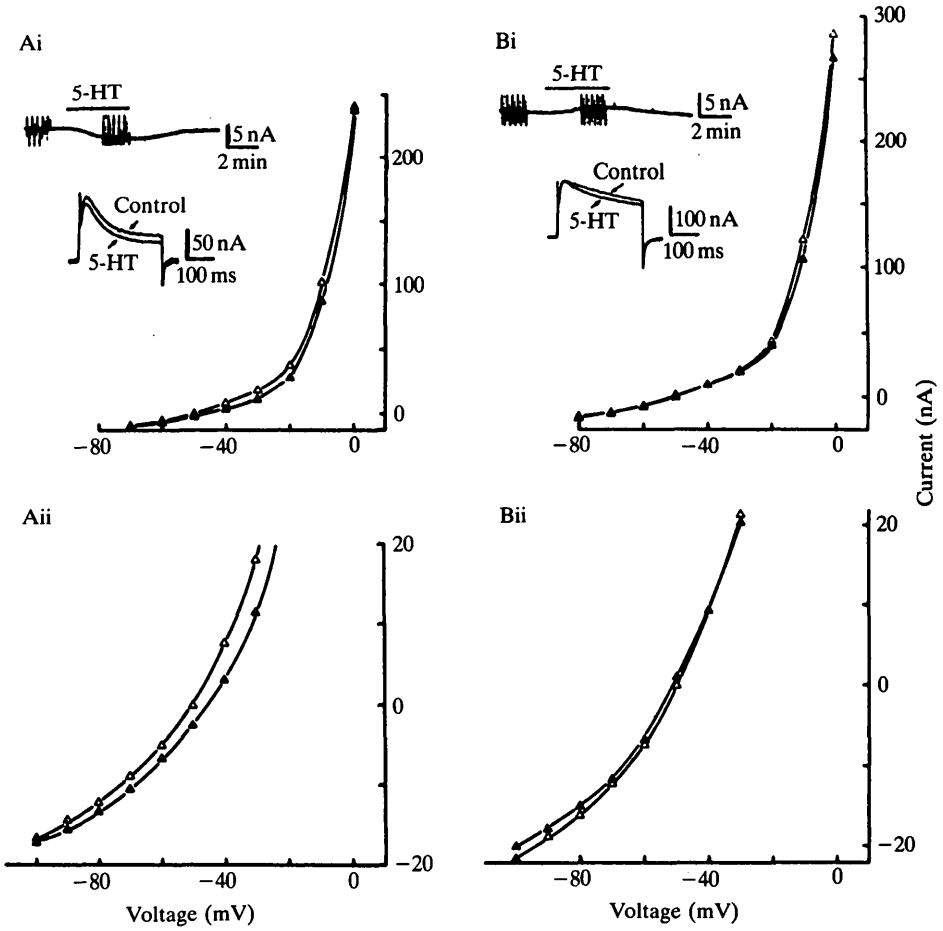

Fig. 4. Effects of 5-HT on the quasi-steady membrane current of PON. Amplitude of current at the end of the command pulse (300 ms duration) was plotted against the command voltage. Holding potential was −50 mV. Open triangles, I–V relationships before application of 3 μmol l^{-1} 5-HT; closed triangles, I–V relationships during application of 3 μmol l^{-1} 5-HT. Vertical deflections in upper insets of Ai and Bi are current signals in response to command pulses at 0·1 Hz. A and B are from the same preparation. All records were made in Na^+-, Ca^{2+}-free solution as described in the text. (Ai) I–V relationships in 3·3 mmol l^{-1} K^+ solution. Upper inset shows the effect of 5-HT on the holding current. Lower inset shows currents in response to the command pulse to −20 mV with and without 5-HT. (Aii) I–V relationships at enlarged vertical scale. (Bi) I–V relationships in 33 mmol l^{-1} K^+ solution. Upper inset shows the effect of 5-HT on the holding current. Lower inset shows currents in response to the command pulse to −10 mV with and without 5-HT. (Bii) I–V relationships at enlarged vertical scale.

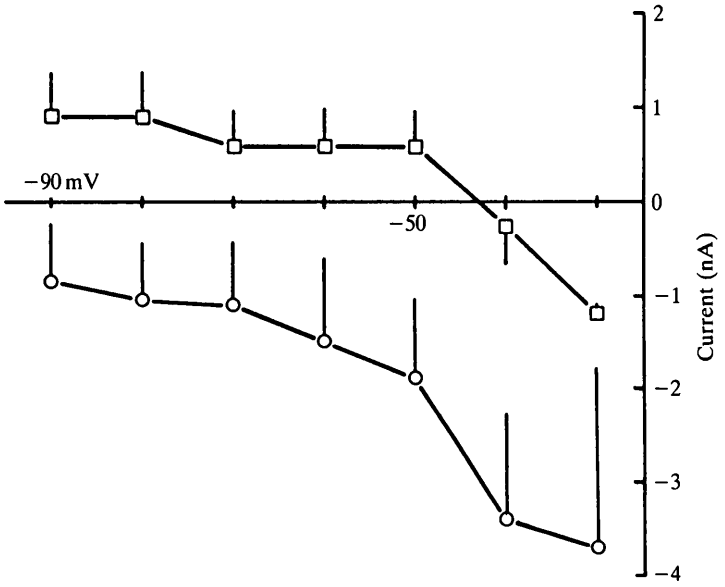

Fig. 5. 5-HT-sensitive current in 3·3 mmol l^{-1} K^+ solution (circles) and 33 mmol l^{-1} K^+ solution (squares). 5-HT-sensitive current was obtained as the difference current in an experiment similar to that in Fig. 4. The amplitude of the mean 5-HT-sensitive currents is plotted against the command voltage; each vertical bar is the S.D. of the mean. $N = 9$ for 3·3 mmol l^{-1} K^+ and $N = 3$ for 33 mmol l^{-1} K^+.

A plot of reversal potential as a function of the logarithm of K^+ concentration, using extrapolation to obtain a value for 3·3 mmol l^{-1} is in good agreement with the Nernst equation (Fig. 6) and suggests that the current is a K^+ current. The results also indicate that the ion channel carrying the 5-HT-sensitive current can function over a wide range of voltages, around the resting level (−50 mV). The current was also found to be blocked by injection of Cs^+ into PON (data not shown), producing further evidence that the current is a K^+ current. These results indicate that the slow depolarization by 5-HT is mainly due to a decrease of K^+ conductance.

5-HT increases Ca^{2+} current in PON

The effects of 5-HT on the active currents were examined in the axotomized PON. This preparation is more suitable than the intact neurone for the purpose because it provides much better conditions for space-clamp.

The membrane currents of axotomized PON, measured using depolarizing command pulses, were characterized by a transient inward current and slowly developing outward current (Fig. 7A). Holding potential was set at −40 mV to inactivate the A current (Thompson, 1977). Peak inward current and delayed outward current were increased by 5-HT (Fig. 7) and the transient outward current (A current), which was seen after turning off the hyperpolarizing command pulse, also showed a slight increase (Fig. 7B). Similar results were

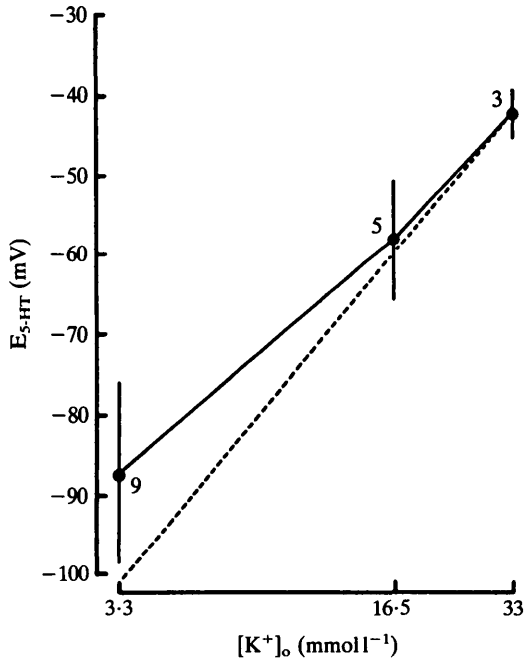

Fig. 6. Effect of $[K^+]_o$ upon reversal potential of 5-HT-sensitive current ($E_{5\text{-HT}}$) in PON. Closed circles are mean values of 3–9 preparations (indicated on figure) and bars are S.D. of the mean. The dotted line represents the change of E_K predicted by the Nernst equation. It was drawn through the mean value at 33 mmol l^{-1} $[K^+]_o$.

obtained in all tested cells ($N = 11$) and the threshold concentration of 5-HT was about 1 μmol l^{-1}.

In the normal physiological solution, outward current increased during the command pulse (see Fig. 7A). In Ca^{2+}-free solution, however, outward current came to a peak followed by a slight decay during the command pulse, and total outward current was greatly reduced (Fig. 8A). These results suggest that the delayed outward current of PON in the normal solution includes a substantial Ca^{2+}-dependent K^+ current (Meech, 1978). 5-HT reduced the outward current and slightly raised the peak inward current in Ca^{2+}-free solution (Fig. 8), indicating that 5-HT lowered the K^+ conductance. Similar results were obtained in three other preparations. It is therefore proposed that 5-HT increases Ca^{2+} current, and that the increased Ca^{2+} influx produces an increase of Ca^{2+}-dependent K^+ current secondarily, although the possibility that 5-HT directly increases the Ca^{2+}-dependent K^+ current remains to be tested.

To investigate further whether 5-HT raised Ca^{2+} current, the effect of 5-HT was examined in TEA^+, Ba^{2+} solution (see Table 1), in which all Na^+ was replaced by TEA^+ to block Na^+ and K^+ conductances. Ba^{2+} does not activate the Ca^{2+}-dependent K^+ current and a larger current can be recorded because the permeability of Ba^{2+} is higher than that of Ca^{2+} in the Ca^{2+} channel (Hagiwara &

Ohmori, 1982). Ba^{2+} is also known to block some K^+ channels (Hille, 1984). In the experiment shown in Fig. 9, 5 mmol l^{-1} 4-aminopyridine (4-AP) was also added to block the A current (Thompson, 1977). In response to the depolarizing steps,

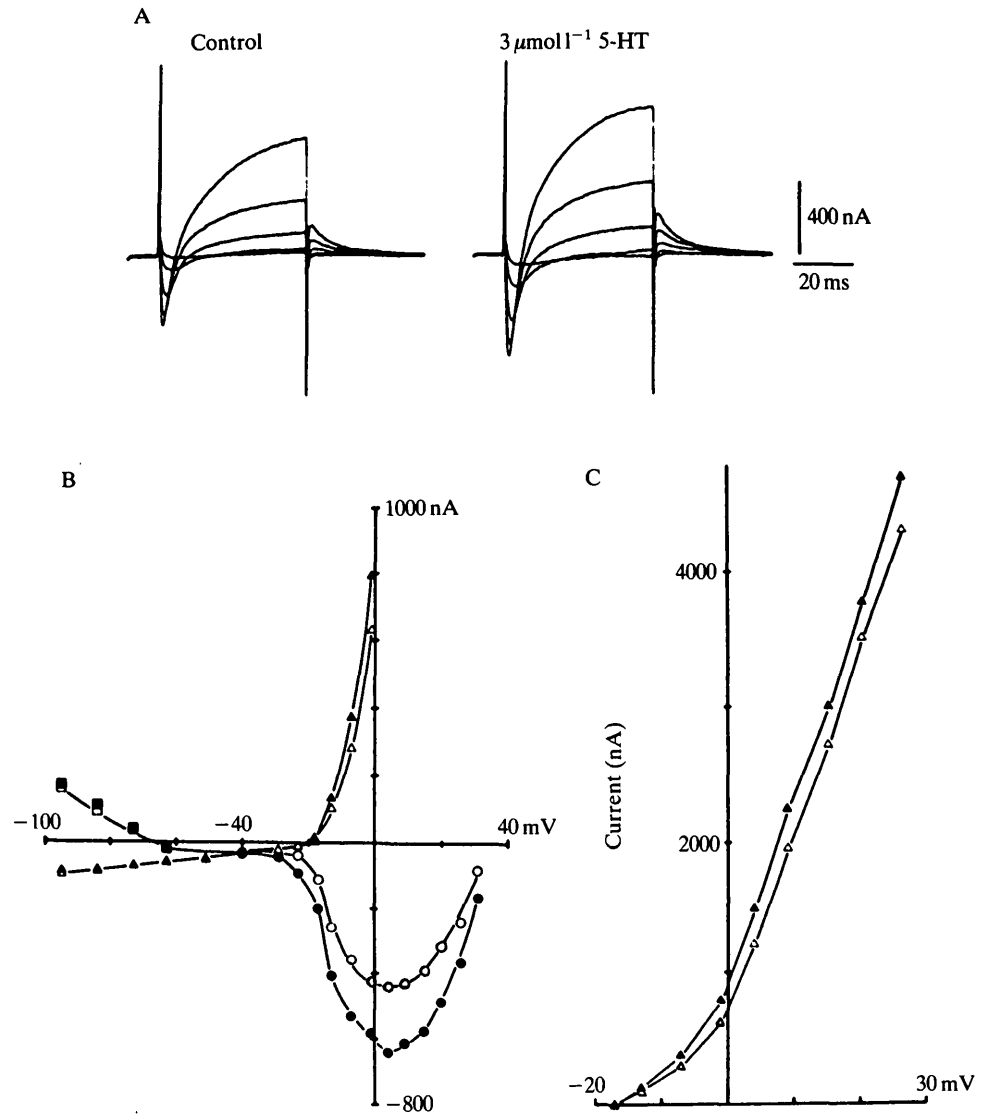

Fig. 7. Effects of 5-HT on the active currents of PON in the normal physiological solution. (A) Membrane currents with and without 3 μmol l^{-1} 5-HT. Holding potential was −40 mV. The command pulses were 50 ms in duration and stepped to −23, −17, −13, −7 and −1 mV. (B) I–V relationships with (closed symbols) and without (open symbols) 3 μmol l^{-1} 5-HT. Circles, peak inward current; triangles, current at the end of the pulse; squares, peak transient outward current activated after the hyperpolarizing pulse. (C) I–V relationships of outward currents measured at the end of the pulse with (closed triangles) and without (open triangles) 3 μmol l^{-1} 5-HT. Some of them (below 0 mV) are plotted in B at enlarged vertical scale.

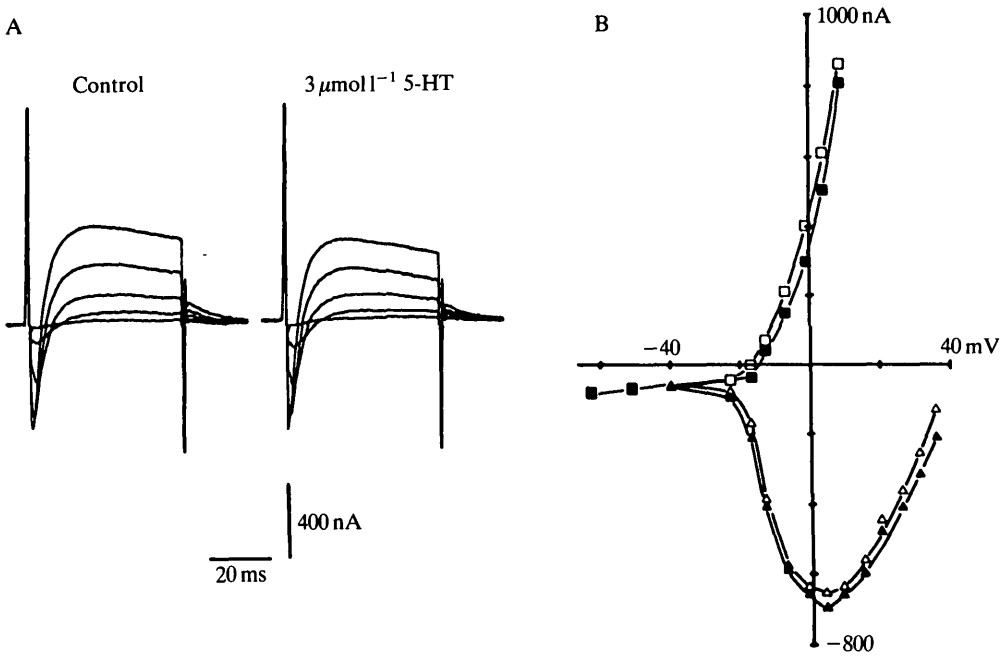

Fig. 8. Effects of 5-HT on the active currents of PON in Ca^{2+}-free solution. (A) Membrane currents with and without 3 μmol l^{-1} 5-HT. Holding potential was −40 mV. The command pulses were 50 ms in duration and stepped to −23, −17, −13, −7 and −1 mV. (B) I–V relationships with (closed symbols) and without (open symbols) 3 μmol l^{-1} 5-HT. Triangles, peak inward current; squares, current at the end of the pulse.

slowly activating Ba^{2+} currents were recorded and these currents showed little inactivation during the command pulse (Fig. 9). This Ba^{2+} current was depressed by the addition of 2 mmol l^{-1} Co^{2+} (not shown), suggesting that this current is carried through the Ca^{2+} channel. The Ba^{2+} current was greatly increased by 5-HT (Fig. 9) and this was confirmed in six other cells. These results indicate that 5-HT increases the voltage-dependent Ca^{2+} current of PON.

5-HT increases the inward rectifying K^+ current

In the axotomized PON, 5-HT was also found to increase another conductance. In the normal physiological solution, 5-HT produced an inward shift in holding current, and the membrane current in response to a hyperpolarizing step was decreased by 5-HT (Fig. 10Ai). However, in 3K solution, the membrane current in response to the same hyperpolarizing step was increased by 5-HT (Fig. 10Aii). The I–V curve showed an inward rectification in 3K solution and this inward rectifying current was increased by 5-HT (Fig. 10B) and depressed by the addition of 1 mmol l^{-1} Ba^{2+} (not shown). These features suggest that this current is an inward rectifying K^+ current (Hagiwara, 1983). This current was not studied further as it was too small in the normal physiological solution.

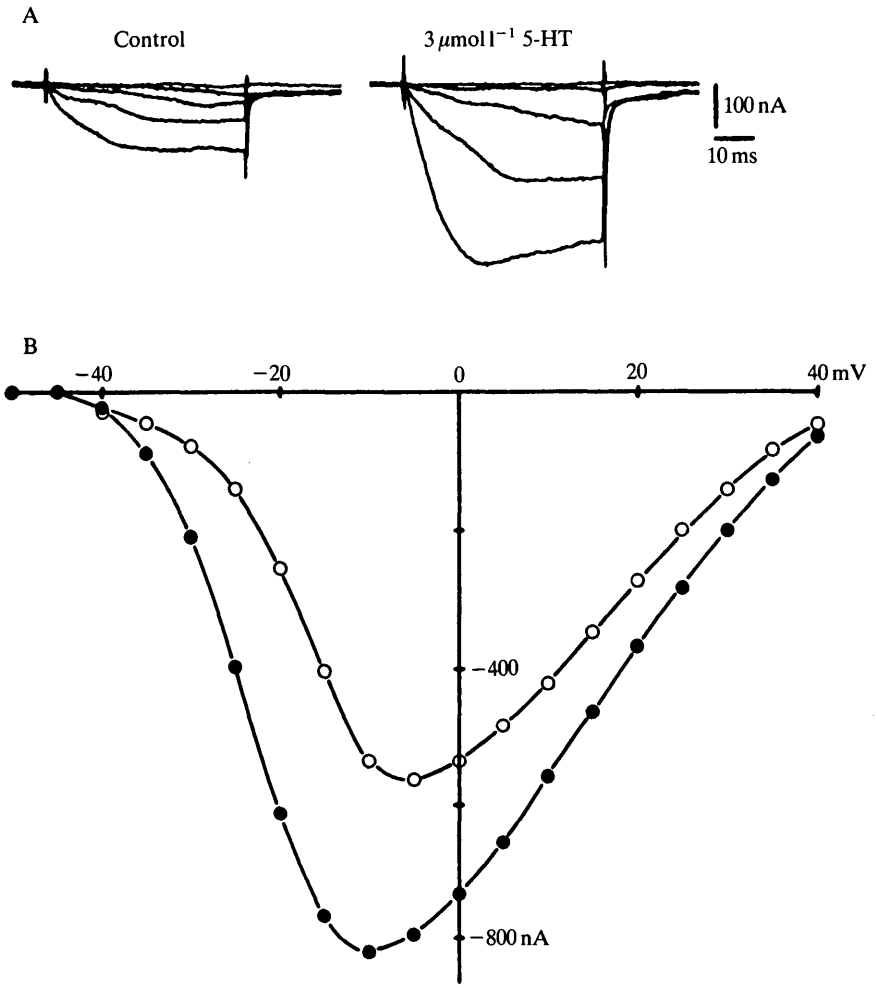

Fig. 9. Effect of 5-HT on the Ba^{2+} current of PON in the TEA^+, Ba^{2+} solution with 5 mmol l^{-1} 4-AP. (A) Ba^{2+} currents with and without 3 μmol l^{-1} 5-HT. Holding potential was −50 mV. The command pulses were 50 ms in duration and stepped to −45, −40, −35, −30 and −25 mV. Capacitive and linear leak currents were subtracted. (B) I–V relationships of the peak Ba^{2+} current with (closed circles) and without (open circles) 3 μmol l^{-1} 5-HT. The slight shift of the I–V relationship along the voltage axis in 5-HT-containing solution is not typical.

Spike broadenings of PON by synaptic action and 5-HT application

A burst of impulses in the cerebral neurones, or application of 5-HT, produced spike broadening in PON (Fig. 11). PON was slightly hyperpolarized to depress spontaneous activity and was made to fire by injection of depolarizing current at 0·5 Hz. The intensity of the current was chosen so that each pulse produced a single spike (multiple spikes in this cell easily induced spike broadening). A burst of impulses in d-LCDN produced a slight depolarization and spike broadening in PON (Fig. 11A). Spike broadening could also result from the depolarization

produced by a constant current injection (Fig. 11B). Thus, it was not clear to what extent the synaptically induced conductance changes were concerned with the spike broadening recorded in the soma. However, synaptic action had another

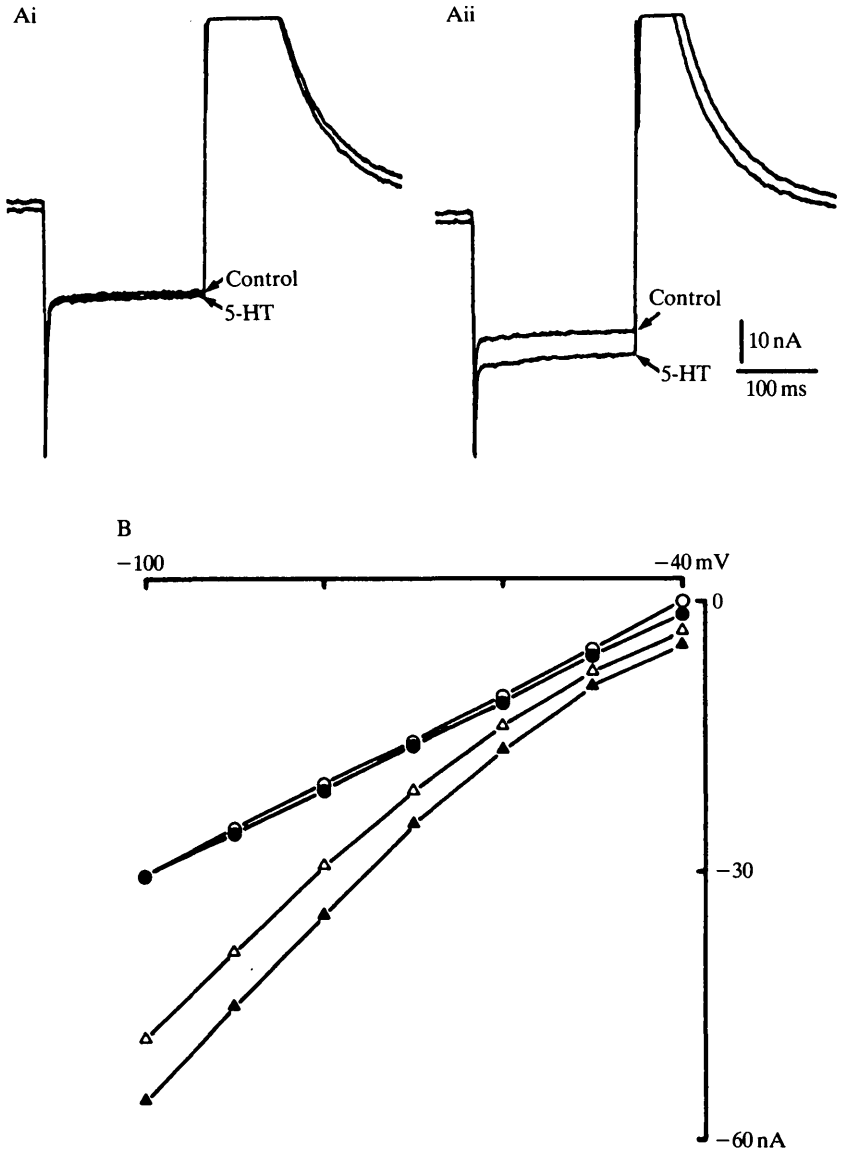

Fig. 10. Effect of 5-HT on the inward rectifying K^+ current of PON. Holding potential was −40 mV and the command pulses were 200 ms in duration. (Ai) Membrane currents in the normal physiological solution, with and without 4 μmol l^{-1} 5-HT. (Aii) Membrane currents in 3K solution, with and without 4 μmol l^{-1} 5-HT. The command pulse was stepped to −80 mV in Ai and Aii. The large A current after the command pulse is clipped in both Ai and Aii. (B) I–V relationships at the end of the pulse, with (closed symbols) and without (open symbols) 4 μmol l^{-1} 5-HT. Circles, currents in the normal physiological solution; triangles, currents in 3K solution.

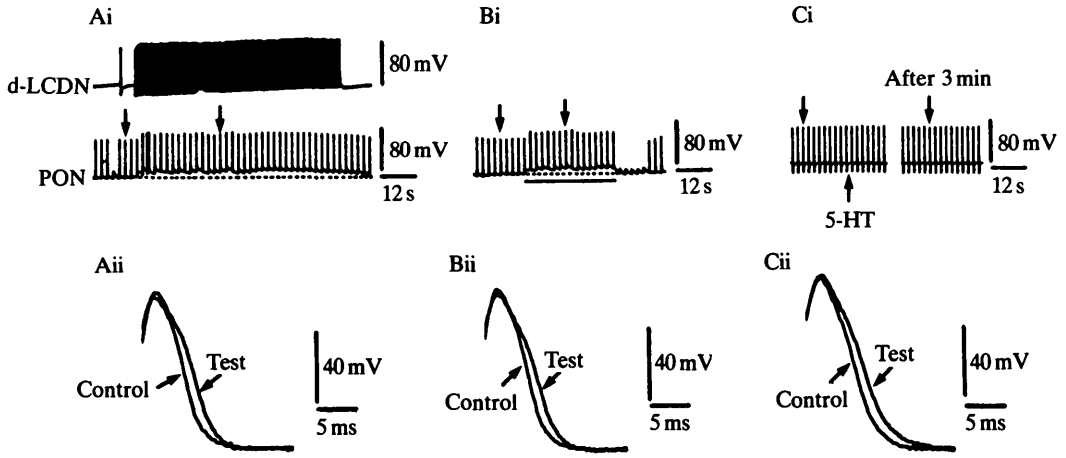

Fig. 11. Spike broadening of PON produced by a burst of impulses in d-LCDN (A), the steady depolarization by a constant current injection (B) or application of 5-HT at 5 μmol l^{-1} (C). PON was hyperpolarized to −50 mV and made to fire by a depolarizing current injection at 0·5 Hz. Dotted lines in Ai and Bi indicate −50 mV level and the bar in Bi indicates the duration of constant depolarizing current injection. Arrows in Ai, Bi and Ci indicate selected spikes which are displayed at expanded time scale in Aii, Bii and Cii.

effect: the excitability of PON was greatly increased by the burst in the cerebral cells. In this experiment, the intensity of injected depolarizing current to PON had to be reduced during the burst of d-LCDN to inhibit the generation of multiple spikes by the injected current. Fig. 11C illustrates the spike broadening of axotomized PON by 5-HT. 5-HT did not depolarize PON at this potential (−50 mV), because the 5-HT-sensitive K^+ conductance was small in the axotomized preparation and became prominent at a more depolarized potential.

Multiple axons of PON and the effect of spike broadening on the conduction in those axons

PON has multiple axons only in the intestinal nerve which goes to the heart (Goto, Ku & Takeuchi, 1986; Furukawa & Kobayashi, 1987*a*). However, it is not known whether all axons go to the heart. In the present study, the existence of multiple axons was tested electrophysiologically. When the soma of PON is hyperpolarized beyond a certain point, an antidromic action potential elicited by stimulation of a branch of the intestinal nerve going to the heart (heart nerve) can be recorded as a depolarizing wave (Furukawa & Kobayashi, 1987*a*). If only one axon of PON goes to the heart, the amplitude of the depolarizing wave recorded at the soma should remain constant when the stimulus intensity is increased. If several axons of PON go to the heart, graded depolarizing waves should be recorded by increasing the stimulus intensity. In the experiment shown in Fig. 12A, at least four different depolarizing waves were discriminated by

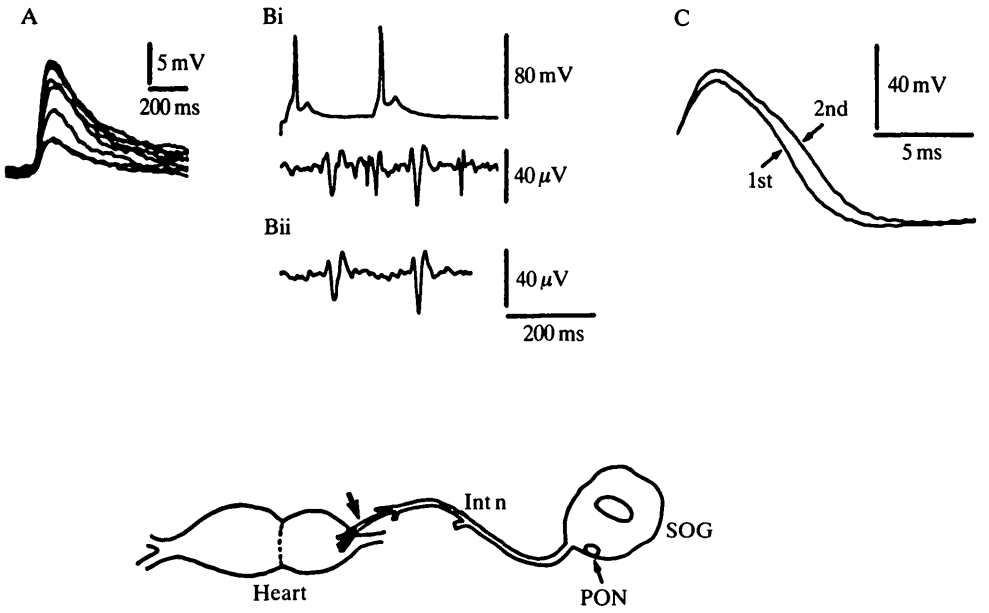

Fig. 12. (A) Superimposed graded depolarizing waves of PON produced by stimulation of the heart nerve. PON was hyperpolarized to prevent generation of a regenerative spike. The duration of stimuli was 0·5 ms and the stimulus intensity was increased from 3 V to 7·5 V by 0·5-V steps. (B) Simultaneous recording from PON and the heart nerve. PON was made to fire twice every 3 s by two successive current injections and the interval of two succeeding spikes was 200 ms. One example of such spikes is shown in Bi. (Bii) Extracellularly recorded spikes of PON averaged from 10 trials such as those shown in Bi. (C) Superimposed soma spikes of PON shown in Bi. The schematic drawing shows the position of the soma of PON and the stimulating or recording point (arrow) in the heart nerve. Int n; intestinal nerve. SOG; suboesophageal ganglion.

increasing the stimulus intensity, indicating the existence of multiple axons of PON in the heart nerve.

Fig. 12B illustrates simultaneous recordings of the membrane potential of PON and extracellular activity of the heart nerve. PON was made to fire at 3-s intervals with a pair of spikes separated by 200 ms. This separation was shown to produce spike broadening at the second spike (see Fig. 12C). Under these conditions, the amplitudes of the extracellularly recorded spikes of PON in the heart nerve were not the same, the second spike being larger than the first (Fig. 12Bi). This was also seen in the averaged signal (Fig. 12Bii). These results suggest that there may be conduction block in some axons of PON, and these failures may be overcome by spike broadening.

Discussion

In the present study, the ionic mechanisms underlying the slow depolarization of PON produced by activity of cerebral neurones (d-LCDN and d-RCDN) and

those underlying the actions of 5-HT upon PON were investigated in the central nervous system of the African giant snail, *Achatina fulica*.

The slow depolarization of PON induced by activity of the cerebral cells was depressed by methysergide. Methysergide is known to be a specific 5-HT antagonist in the molluscan central nervous system (Leake & Walker, 1980). 5-HT induced a similar slow depolarization in the axotomized PON which was also depressed by methysergide. Furthermore, the ionic mechanisms underlying both types of depolarization were found to be similar. These results suggest that the neurotransmitter of the two cerebral neurones may be 5-HT.

Activity of the cerebral cells produced an inward shift in the current required to hold the membrane potential of PON at the resting level, together with a decrease in K^+ conductance. Thus, the current which was decreased by activity of the cerebral cells contributes to the resting potential of PON, i.e. this K^+ current is the background K^+ current. A similar synaptically mediated decrease of the background K^+ conductance has been reported in sensory neurones of *Aplysia* (Klein & Kandel, 1980).

In some preparations, activity of the cerebral cells produced an increase of conductance in PON, preceding the decrease of K^+ conductance. This increased conductance is considered to be a Ca^{2+} conductance for the following reasons. (1) The slow depolarization of PON was not depressed by replacing Na^+ with impermeant $Tris^+$. (2) Injection of Cl^- into PON had no effect on the slow depolarization. (3) Injection of EGTA into PON increased the peak amplitude of the apparent inward current. Although these results are not straightforward, an increase of Ca^{2+} conductance seems a likely explanation.

The slow depolarization induced by 5-HT was mainly due to a decrease of K^+ conductance which was also chiefly responsible for the background K^+ current. Thus, the current which is sensitive to 5-HT is quite similar to the K^+ current which is decreased by activity of the cerebral cells. The 5-HT-sensitive K^+ current is not Ca^{2+}-dependent as it could be recorded in Ca^{2+}-free solution containing Co^{2+}. Because the 5-HT-sensitive K^+ current of PON was seen over a wide voltage range around the resting level and showed little time-dependency, it is neither the delayed rectifying K^+ current nor the A current. 5-HT-induced closure of a certain K^+ channel which has similar properties to the 5-HT-sensitive K^+ channel of PON has been reported in some molluscan neurones (Siegelbaum *et al.* 1982; Paupardin-Tritsch *et al.* 1981; Jacklet & Acosta-Urquidi, 1985).

The 5-HT-sensitive K^+ current of PON could not be reversed at an external K^+ concentration of $3{\cdot}3\,mmol\,l^{-1}$ (standard K^+ concentration of the normal physiological solution for *Achatina*). Indeed, reversal could not be obtained until $[K^+]_o$ was raised more than fivefold. The likely explanation seems to be constant field rectification by the uneven distribution of K^+ across the membrane, i.e. measurable inward current cannot flow in the normal K^+ gradient (Siegelbaum *et al.* 1982; Pollock, Bernier & Camardo, 1985), although the possibility that this K^+ channel has an outward rectifying property cannot be ruled out.

5-HT also increased the voltage-dependent Ca^{2+} current in the axotomized PON. It is possible that this modulation of Ca^{2+} current by 5-HT is related to the presumed increase of Ca^{2+} conductance of PON caused by a burst of impulses from the cerebral cells. 5-HT-induced increase of Ca^{2+} current is now known to exist in some molluscan neurones (Paupardin-Tritsch *et al.* 1986*b*; Jacklet & Acosta-Urquidi, 1985) and may exist in vertebrate neurones (Hounsgaard & Kiehn, 1985). Modulation of Ca^{2+} current in the neuronal membrane is important; e.g. it is considered to underlie several plasticities of synapses (Klein, Shapiro & Kandel, 1980).

In 3K solution, substantial inward rectifying K^+ current was revealed in PON, and this K^+ current was also increased by 5-HT. Enhancement of an inward rectifying K^+ current by 5-HT has been reported in *Aplysia* Rl5 (Benson & Levitan, 1983) which is considered to be a homologous neurone to PON (Furukawa & Kobayashi, 1987*a*).

Depression of the background K^+ conductance of PON should lead to an increase in excitability. As reported previously, PON is the most effective heart excitor in *Achatina*, but its activity is usually reduced by many inhibitory synaptic inputs (Furukawa & Kobayashi, 1987*a*). Thus, increased excitability of this cell should have significant effects upon heart regulation of this snail.

Activity of the cerebral cells or application of 5-HT produced spike broadening in PON, as was expected from the results of the voltage-clamp experiments. This spike broadening may also have a role in heart regulation. The physiological significance of spike broadening has been thoroughly studied in *Aplysia* sensory neurones (Klein & Kandel, 1978; Hochner, Klein, Schacher & Kandel, 1986). In *Aplysia*, spike broadening of sensory neurones produced by connective stimuli or by application of 5-HT results in an increase of EPSPs in its follower cell, the gill motoneurone, probably through increased Ca^{2+} influx at the terminal during the broadened spike. Such modulation of synaptic efficacy may also occur at the terminals of PON, although the great distance from the ganglia to the heart (usually more than 3 cm) makes a deduction from the phenomena recorded in the soma difficult.

It was also demonstrated in this study that at least some of the multiple axons of PON reach the heart, and the spike broadening in the soma of PON appeared to correlate with an increased amplitude of PON spikes in the heart nerve, recorded extracellularly. This may be because the multiple axons have different thresholds, so that the spike broadening recruits more axons. If this hypothesis is correct, the number of terminals activated by a single somatic action potential would be increased by spike broadening, which would augment the heart excitatory action of PON. To test this hypothesis, a quantitative investigation of the relationship between the width of the spike of PON and its heart excitatory action would be necessary.

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