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MODULATORY EFFECTS OF 5-HYDROXYTRYPTAMINE ON VOLTAGE-ACTIVATED CURRENTS IN CULTURED ANTENNAL LOBE NEURONES OF THE SPHINX MOTH *MANDUCA SEXTA*

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Summary

The modulatory effects of 5-hydroxytryptamine (5-HT or serotonin) on voltage-gated currents in central olfactory neurones of the moth *Manduca sexta* have been examined *in vitro* using whole-cell patch-clamp recording techniques. Central olfactory neurones were dissociated from the antennal lobes of animals at stage 5 of the 18 stages of metamorphic adult development. The modulatory actions of 5-HT on voltage-activated ionic currents were examined in a subset of morphologically identifiable antennal lobe neurones maintained for 2 weeks in primary cell culture. 5-HT caused reversible reduction of both a rapidly activating A-type K^+ current and a relatively slowly activating K^+ current resembling a delayed rectifier-type conductance. 5-HT also reduced the magnitude of voltage-activated Ca^{2+} influx in these cells. The functional significance of 5-HT-modulation of central neurones is discussed.

Key words: 5-hydroxytryptamine, serotonin, *Manduca sexta*, neuromodulation, antennal lobe, olfaction, cell culture.

Introduction

The first central olfactory processing station of the insect brain, the antennal lobe (AL), bears a striking morphological resemblance to the analogous structure in the vertebrate brain, the olfactory bulb. Both the AL and the olfactory bulb exhibit a central region of coarse neurites that is surrounded by spheroidal regions of densely packed synaptic neuropile called glomeruli. In each system, the glomeruli contain the terminal arborizations of primary afferent axons, processes of local interneurones, neurites of projection (output) neurones and ramifications of centrifugal neurones from other regions of the brain (see Homberg *et al.* 1989; Shepherd *et al.* 1987). Furthermore, both the AL and the olfactory bulb receive efferent centrifugal input from neurones that contain the biogenic monoamine 5-hydroxytryptamine (5-HT or serotonin; Halász and Shepherd, 1983; Kent *et al.* 1987; Soghomoniam *et al.* 1988; Homberg *et al.* 1989; Salecker and Distler, 1990; Sun *et al.* 1992, 1993). In neither system, however, is the functional significance of 5-HT well understood. We have examined the modulatory effects of 5-HT on central olfactory neurones of the sphinx moth, *Manduca sexta*, using AL neurones in primary cell culture as an experimentally tractable system for investigations of the mechanisms of action of 5-HT.

The organization, development and neurochemistry of the ALs have been examined in considerable detail in *M. sexta* (e.g. Sanes and Hildebrand, 1976*a*,*b*; Tolbert and Hildebrand, 1981; Schneiderman *et al.* 1982; Tolbert *et al.* 1983; Hildebrand, 1985; Hoskins *et al.* 1986; Kent *et al.* 1987; Homberg and Hildebrand, 1989; Homberg *et al.* 1988, 1989), and single-unit recording and staining of central olfactory neurones have been used extensively to study the processing of olfactory information in the brain of this species (e.g. Matsumoto and Hildebrand, 1981; Christensen and Hildebrand, 1987*a*,*b*; Kanzaki *et al.* 1989, 1991*a*,*b*; Hansson *et al.* 1991).

Each of the two ALs in the brain of the moth receives input from a single 5-HT-immunoreactive neurone (5-HT-IRN), the cell body of which is located in the contralateral AL (Kent *et al.* 1987; Homberg *et al.* 1989). In the brain of the adult moth, each 5-HT-IRN projects to most, if not all, glomeruli of the AL it innervates. Synaptic contacts between the 5-HT-IRN and other neurones in the glomerular neuropile of the AL are predominantly output synapses from the 5-HT-IRN (Sun *et al.* 1992, 1993). Although the identities of cells postsynaptic to the 5-HT-IRN have yet to be determined, intracellular studies have shown that the responses of some AL neurones to primary afferent synaptic input can be modulated by 5-HT. When

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applied at 10^{-4} mol l^{-1}, 5-HT increases cell excitability, leads to a broadening of action potentials in some neurones and increases cell input resistance (Kloppenburg and Hildebrand, 1992, 1995).

The 5-HT-IRN associated with each AL can be identified as early as the first larval instar (Kent *et al.* 1987). The close association of these neurones with the ALs throughout metamorphic adult development is consistent with the possibility that 5-HT contributes to the developmental regulation of central olfactory neurones in the moth. In the present investigation, we have taken advantage of procedures recently developed for long-term maintenance of *M. sexta* central olfactory neurones in primary cell culture (Hayashi and Hildebrand, 1990) to explore the modulatory actions of 5-HT on AL neurones from animals early (stage 5) in metamorphic adult development. We have used whole-cell patch-clamp recording techniques to examine *in vitro* the effects of 5-HT on voltage-activated membrane currents in a morphologically identifiable subset of AL neurones that show highly consistent responses to 5-HT. These neurones have been referred to elsewhere as RR neurones (Oland *et al.* 1992; Oland and Hayashi, 1993). 5-HT exerts multiple effects on these cells, the possible functional significance of which is discussed.

A preliminary report of some of this work has appeared elsewhere (Mercer *et al.* 1992).

Materials and methods

Insects

Manduca sexta (Lepidoptera: Sphingidae) were reared on an artificial diet (modified from that of Bell and Joachim, 1976) and maintained at 25 °C under a long-day photoperiod regimen (17 h:7 h light:dark) and 50–60 % relative humidity. *M. sexta* larvae hatch from eggs and pass through five instars prior to pupation. Metamorphic adult development proceeds through 18 stages, each of which is accompanied by well-defined changes in pupal morphology (Sanes and Hildebrand, 1976*a*,*b*; Tolbert *et al.* 1983). Moths at stage 5 of metamorphic adult development were examined in this study.

Preparation of cultures

Techniques used to culture *M. sexta* AL neurones have been described previously (Hayashi and Hildebrand, 1990). Brains were removed from cold-anaesthetized pupae and placed into sterile culture saline containing (in mmol l^{-1}) 149.9 NaCl, 3 KCl, 3 $CaCl_2$, 0.5 $MgCl_2$, 10 Tes and 11 D-glucose, as well as 6.5 g l^{-1} lactalbumin hydrolysate, 5 g l^{-1} TC yeastolate (Difco), 10 % foetal bovine serum (FBS), 100 i.u. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, adjusted to pH 7 and 360 mosmol l^{-1}. ALs were transferred into Hanks' Ca^{2+}- and Mg^{2+}-free buffered salt solution containing 0.5 mg ml^{-1} collagenase and 2 mg ml^{-1} Dispase for 2 min at 37 °C to dissociate the tissue, which was then dispersed by trituration with a fire-polished Pasteur pipette. Enzyme treatment was terminated by centrifuging cells, first through 6 ml of culture saline and then through the same volume of culture medium (composition below). Dissociated cells were allowed to settle and to adhere to the surface of culture dishes coated with Concanavalin A (200 μg ml^{-1}) and laminin (2 μg ml^{-1}). Cultures were placed in a humidified incubator at 26 °C. The culture medium was replaced every 3–4 days, and cells were maintained *in vitro* for 2–3 weeks.

Culture medium

The following additions were made to 500 ml of Leibovitz's L15 medium (Gibco): 10 % FBS, 185 mg of α-ketoglutaric acid, 200 mg of fructose, 350 mg of glucose, 335 mg of malic acid, 30 mg of succinic acid, 1.4 g of TC yeastolate, 1.4 g of lactalbumin hydrolysate, 0.01 mg of niacin, 30 mg of imidazole, 100 μg ml^{-1} streptomycin, 100 i.u. ml^{-1} penicillin, 1 μg ml^{-1} 20-hydroxyecdysone (Sigma) and 2.5 ml of stable vitamin mix. A 5 ml stock solution of vitamin mix consists of: 15 mg of aspartic acid, 15 mg of cystine, 5 mg of β-alanine, 0.02 mg of biotin, 2 mg of vitamin B12, 10 mg of inositol, 10 mg of choline chloride, 0.05 mg of lipoic acid, 5 mg of *p*-aminobenzoic acid, 25 mg of fumaric acid, 0.4 mg of coenzyme A, 15 mg of glutamic acid and 0.5 mg of Phenol Red. The medium was adjusted to pH 7 and 350 mosmol l^{-1} and filter-sterilized prior to use.

Identification of cells in culture

After 7–10 days, AL neurones in culture can be identified on the basis of their morphology and whole-cell current profile (Hayashi and Hildebrand, 1990; Hayashi *et al.* 1992; Oland *et al.* 1992; Oland and Hayashi, 1993). In the present investigation, cells were maintained for 12–14 days in culture prior to use. Modulatory effects of 5-HT were examined in detail in a subset of AL neurones referred to elsewhere as RR neurones (Oland *et al.* 1992; Oland and Hayashi, 1993). Although the specific identity of these neurones has yet to be established, several lines of evidence suggest that RR neurones are more likely to be local interneurones than projection (output) neurones of the AL (Oland and Hayashi, 1993). The effects of 5-HT on these cells are highly consistent and reversible (Mercer *et al.* 1992).

Biophysical measurements

Membrane currents from AL neurones grown in culture for 12–14 days were recorded using the whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981; Fenwick *et al.* 1982). Electrodes with resistances of 2–4 MΩ were made from borosilicate glass using a Narishige PP-83 electrode puller and were filled with a solution containing 150 mmol l^{-1} potassium aspartate, 10 mmol l^{-1} NaCl, 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $CaCl_2$, 11 mmol l^{-1} EGTA and 2 mmol l^{-1} ATP, and adjusted to pH 7 with Hepes (5 mmol l^{-1}) and to 330 mosmol l^{-1} with mannitol. Cells were visualized using an inverted microscope equipped with Hoffman modulation contrast optics to aid placement of the electrode on the cell soma. To facilitate the formation of high-resistance (gigaohm) seals, culture medium was replaced with insect saline solution (100 mmol l^{-1} NaCl, 4 mmol l^{-1} KCl, 6 mmol l^{-1} $CaCl_2$,

5 mmol l^{-1} D-glucose, 10 mmol l^{-1} Hepes, pH 7), adjusted to 360 mosmol l^{-1} with mannitol prior to recording. Cells were continuously superfused with fresh saline solution throughout the recording period. For whole-cell recording, light suction and brief high-voltage pulses were used to rupture the cell membrane beneath the recording electrode. Membrane currents were recorded using an Axopatch 1B amplifier (Axon Instruments, Foster City, CA). Data were acquired and analyzed with the aid of pClamp software (Axon Instruments) run on an i30386 microcomputer. Cells were clamped at a holding potential of −70 mV, and depolarizing voltage steps were used to activate voltage-gated channels in the cells. Membrane currents were filtered with a 10 kHz low-pass four-pole Bessel filter and sampled at intervals of 100 μs. Junction potentials were nullified prior to seal formation, and linear leakage currents were subtracted electronically from all records. Because no compensation was made for series resistance, voltage errors may be present where currents measured were large. The currents recorded in these cells seldom exceeded 2 nA, and the series resistance (calculated from the capacitative charging transient) was always less than 5 MΩ, suggesting a maximum voltage error of 10 mV. Series-resistance errors should not affect the central conclusions of this study.

Components of the whole-cell current recorded in AL neurones were isolated using routine pharmacological techniques. Sodium currents in *M. sexta* neurones can be blocked with tetrodotoxin (TTX, 0.01 μmol l^{-1}) and calcium currents with 500 μmol l^{-1} $CdCl_2$ (Hayashi *et al.* 1992; Hayashi and Levine, 1992). A-type K^+ currents (Connor and Stevens, 1971) were blocked with 4-aminopyridine (4-AP, 5 mmol l^{-1}; see Rudy, 1988) or by replacing potassium aspartate in the electrode with CsCl (see Armstrong and Bezanilla, 1973). Tetraethylammonium chloride (TEA^+, 30 mmol l^{-1}) was used to block non-A-type K^+ currents. Stable recordings from 3–5 neurones were obtained for each experimental protocol. The results presented in this paper are based on recordings from a total of 53 AL neurones.

5-HT application

Whole-cell current profiles recorded prior to placement of the 5-HT-containing pipette near the cell soma were compared with currents elicited during the application of 5-HT (as 5-HT creatinine sulphate; Sigma) and at several time points thereafter. 5-HT (10, 50, 100 or 500 μmol l^{-1}) was pressure-ejected across the cell body in a pulse beginning 5 ms prior to the onset of each voltage step. Unless otherwise indicated, 5-HT was applied for 30 ms. A visible dye (Neutral Red) added to the 5-HT solution was used in a set of preliminary experiments to establish the optimal placement of the 5-HT delivery pipette (tip diameter approximately 4 μm) and a suitable ejection pressure (approximately 2×10^4 Pa). Increasing the length of the 5-HT pulse did not alter the nature of the effects observed but did affect their magnitude. The dose-dependence of the effects of 5-HT was confirmed by application of two different concentrations of 5-HT to the same cell from puffer pipettes positioned equidistant from the cell body. Brief pulses of 5-HT were used in these experiments to ensure that full recovery from the effects of 5-HT would be observed during the recording session, so that the reproducibility of the effects could also be tested. If long pulses (200–500 ms or greater) of 5-HT were applied to the cells, prolonged washing (⩾20 min) in 5-HT-free saline was required for complete reversal of some of the effects of 5-HT.

Statistical analysis

Within-group and between-group comparisons were made using *t*-tests for correlated or independent means, respectively. Data are expressed as means ± S.E.M.

Results

Whole-cell current profiles of several of the morphologically distinct types of cultured AL neurones described previously (Hayashi and Hildebrand, 1990; Hayashi *et al.* 1992; Oland *et al.* 1992; Oland and Hayashi, 1993), including proximal-branching cells (Fig. 1A), symmetrical cells (Fig. 1B), RR cells (Fig. 1C) and radial cells (Fig. 1D), were reversibly altered by pulse application of 5-HT. In each of these cell types, 5-HT reduced the magnitude of outward current in a manner similar to that shown in Fig. 2. Several unidentified subtypes of neurones did not respond to pulse application of 5-HT.

Modulatory actions of 5-HT were examined in detail in the subset of AL neurones known as RR cells (Fig. 1C). *In vitro*, these cells possess a large and irregularly shaped cell body and neurites that have a characteristic zigzag appearance (Oland and Hayashi, 1993). Whole-cell current profiles in RR neurones taken from animals at stage 5 in their metamorphic adult development were dominated by large outward currents (Fig. 2A). Typically, 5-HT reduced the magnitude of whole-cell current in these cells (Fig. 2B), an effect that reversed as 5-HT was washed away by superfusion with normal saline solution (Fig. 2C). Changing the length of the 5-HT pulse from 5 to 55 ms altered the magnitude of 5-HT-induced effects (Fig. 2D). To determine whether this could be related to changes in the final concentration of 5-HT arriving at the cell surface, different concentrations of 5-HT were applied to the same cell from two independent puffer pipettes. The pipettes had similar tip diameters (approximately 4 μm) and were positioned equidistant from the cell body. Identical pressure pulses were applied to each pipette. Subtracting current profiles recorded in the presence of 5-HT (Fig. 2B) from those observed prior to 5-HT application (Fig. 2A) provides an indication of current blocked by 5-HT (Fig. 2E). Different concentrations of 5-HT applied to the same cell confirmed the dose-dependent nature of the effects of 5-HT (compare Fig. 2E and 2F). This was further confirmed by comparing the percentage reduction of peak outward current in cells treated with low (10–50 μmol l^{-1}) or high (100–500 μmol l^{-1}) concentrations of 5-HT (Fig. 3A). 5-HT also increased

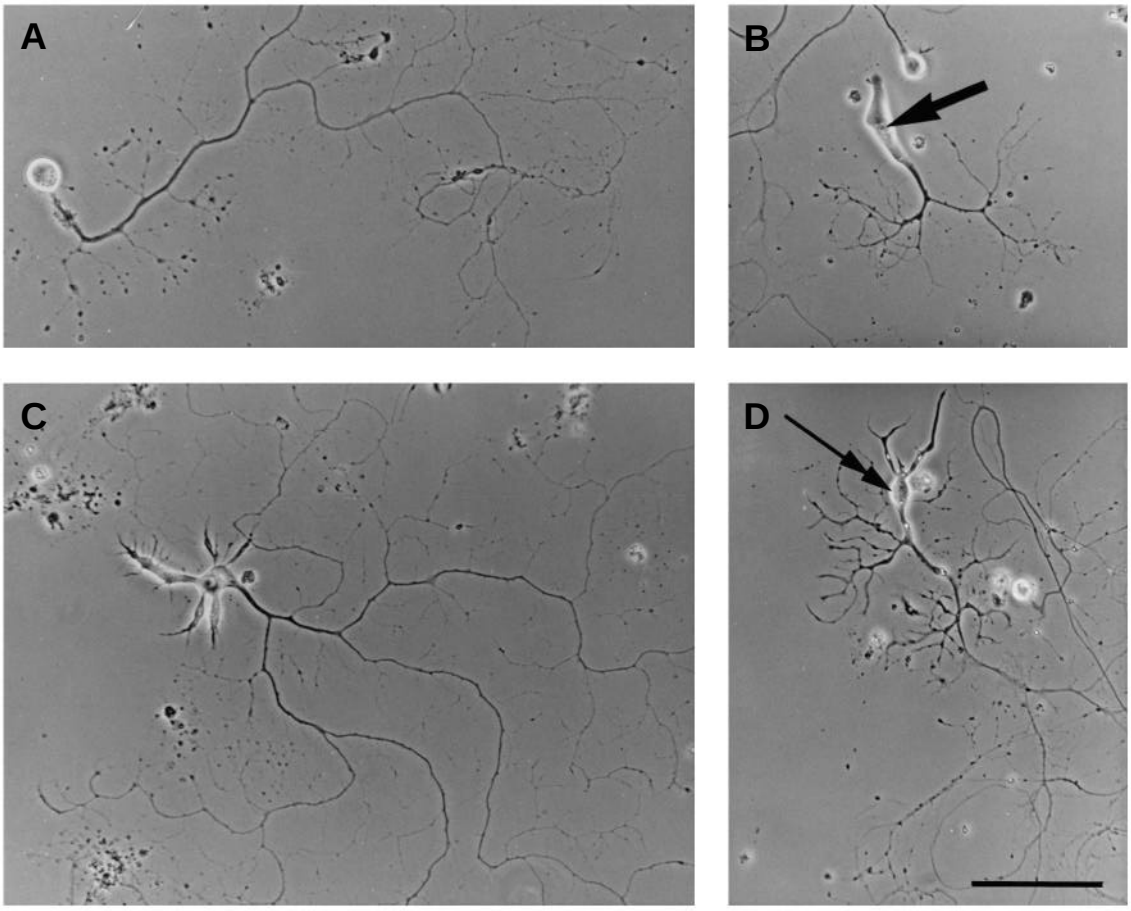

Fig. 1. *In vitro* morphology of four subtypes of *Manduca sexta* antennal lobe neurones, the whole-cell current profiles of which are modulated by 5-HT. (A) Proximal-branching cell, (B) symmetrical cell (arrow), (C) RR cell and (D) radial cell (double-headed arrow). These types of cells have been described elsewhere (Oland and Hayashi, 1993). In this paper, 5-HT-modulation of ionic currents is examined in detail in the subtype of AL neurones known as RR cells. Scale bar, 100 μm.

significantly the time taken to reach peak current levels (Fig. 3B). In 36 % of the cells, 5-HT altered the holding current at −70 mV, indicating that 5-HT might cause a slight depolarization of the membrane of some AL neurones. The effects of 5-HT on non-voltage-gated channels have not been examined further in this study. To examine the effects of 5-HT on voltage-activated currents, components of the whole cell current were isolated using routine pharmacological techniques.

Isolation of potassium currents

Subtraction currents (Fig. 2E,F) suggest that 5-HT affects a fast transient component, as well as a more slowly activating component, of the whole-cell current profile. To examine this further, K^+ currents in *M. sexta* neurones were revealed by blocking Ca^{2+} currents with 500 μmol l^{-1} $CdCl_2$ and Na^+ currents with 10^{-8} mol l^{-1} TTX (Hayashi *et al.* 1992; Hayashi and Levine, 1992). Although functional Na^+ channels do not appear to be expressed in RR cells taken from animals at stage 5 in adult development, Na^+ currents have been observed in cells of this type taken from stage 9 pupae (Mercer *et al.* 1992). The K^+ channel blockers 4-aminopyridine (4-AP, 5 mmol l^{-1}) and tetraethylammonium (TEA^+, 30 mmol l^{-1}) were used to isolate individual components of the macroscopic K^+ current in these cells. The actions of TEA^+ reduced the amplitude but not the general character of the outward current (Fig. 4A). However, 4-AP was more selective than TEA^+ and segregated fast and slow components of the outward current (Fig. 4B). Moreover, the effects of 4-AP were reversed more readily than those of TEA^+ with washing (Fig. 4B). At a concentration of 5 mmol l^{-1}, 4-AP blocked the fast, transient (A-type) K^+ current, leaving the slower-activating component largely intact (Fig. 5). Higher concentrations of 4-AP (10 mmol l^{-1}) were less selective, causing some decrease in the sustained component as well as blocking the fast, transient component of the macroscopic K^+ current in these cells (not shown).

The differential effect of 4-AP on fast and slow components of the outward current is apparent from whole-cell current profiles measured before (Fig. 5A) and after (Fig. 5B) the application of 4-AP. The magnitude of the early, transient current is reduced significantly, whereas the slower component remains largely intact. To reveal the time course and

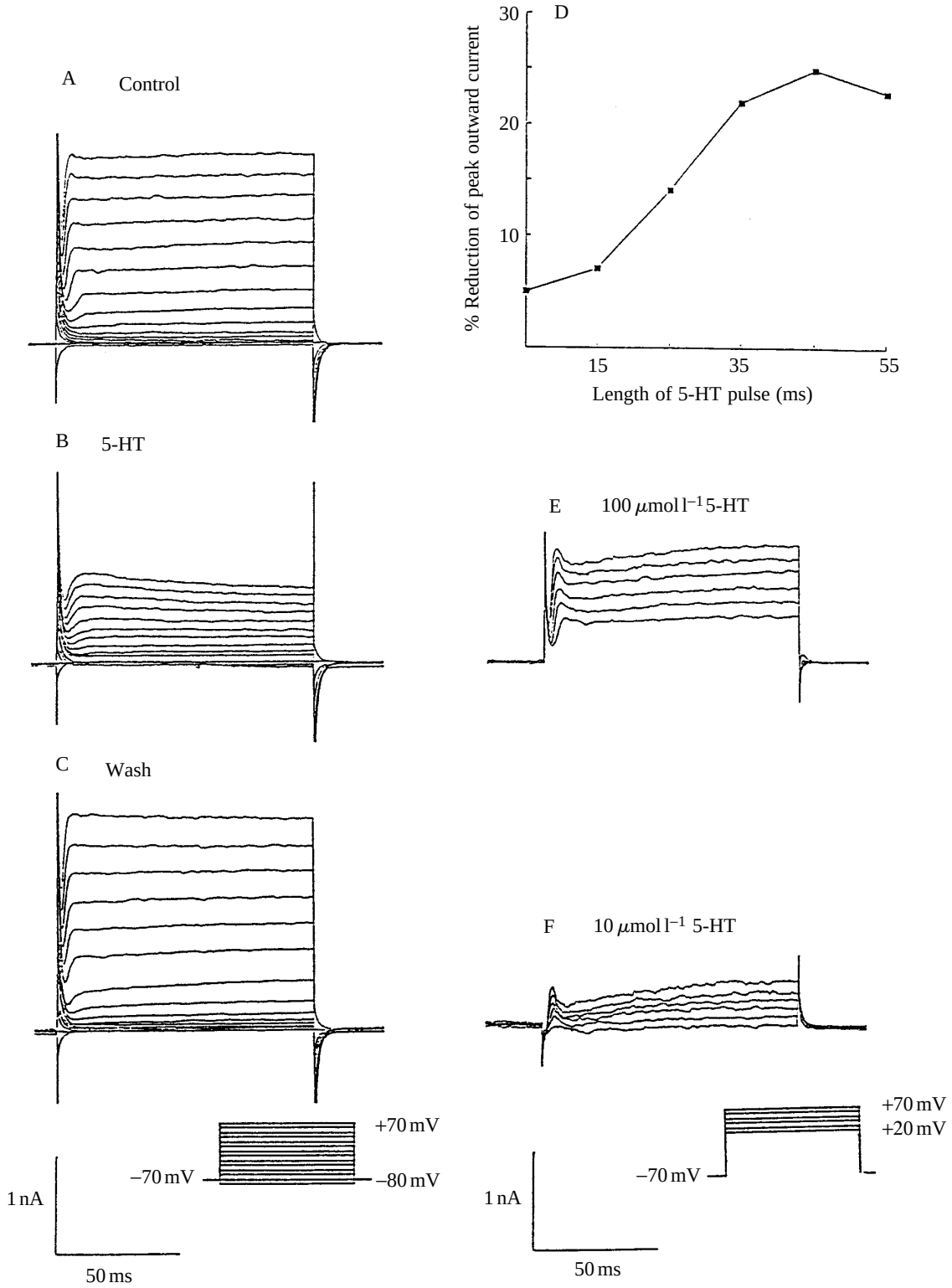

Fig. 2. Whole-cell currents and their reduction by 5-HT. (A) Whole-cell current profiles measured in RR cells from *Manduca sexta* at stage 5 of pupal development. (B) Reduction of whole-cell current by 100 ms pulses of 5-HT (100 μmol l^{-1}). (C) Recovery of the magnitude of outward current after wash-out of 5-HT. (D) Change in the percentage reduction of peak outward current resulting from a stepwise increase in the duration of a 10 μmol l^{-1} pulse of 5-HT from 5 to 55 ms. Increasing pulse duration further had no additional effect on the magnitude of the macroscopic current profile. (E) Difference currents obtained by subtracting current profiles obtained in the presence of 5-HT from those obtained prior to 5-HT application show the magnitude and time course of current blocked by 100 μmol l^{-1} 5-HT. (F) Difference currents to show current blocked by 10 μmol l^{-1} 5-HT in the same cell as in E. Comparison of E and F illustrates the dose-dependence of the effects of 5-HT.

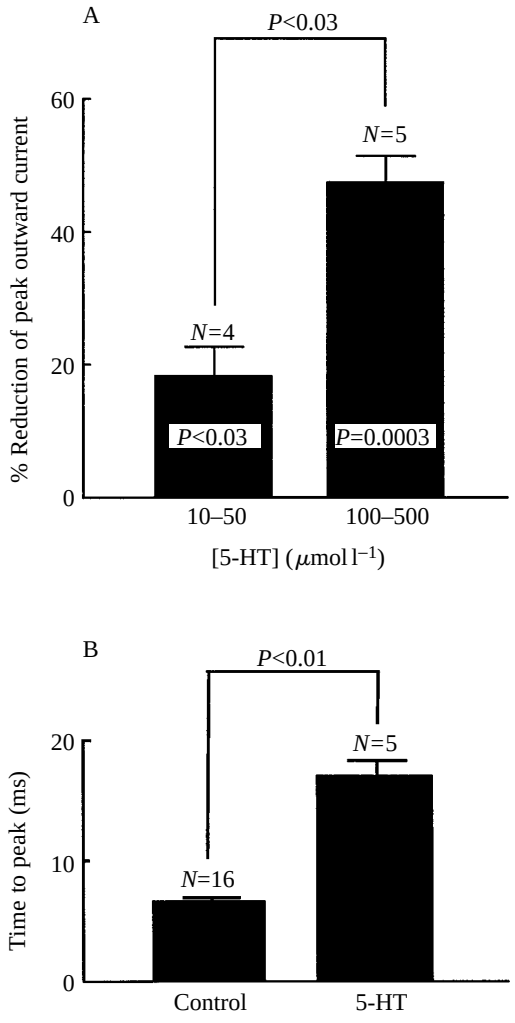

Fig. 3. (A) Percentage reduction of peak outward current by 5-HT. 5-HT caused a significant reduction of peak outward current, at concentrations of 10–50 μmol l^{-1} (P<0.03 using two-tailed t-test) and 100–500 μmol l^{-1} (P<0.0003 using two-tailed t-test). A between-group analysis reveals that the effects of 5-HT at concentrations of 100–500 μmol l^{-1} are significantly greater than the effects of 10–50 μmol l^{-1} 5-HT (P<0.03 using one-tailed t-test). (B) Comparison of time to reach peak current levels following single voltage steps from −70 to +50 mV in the presence and absence of 5-HT (100–500 μmol l^{-1}). A between-group analysis indicates that the time taken to reach peak current levels is significantly greater in the presence of 5-HT (P<0.01 using two-tailed t-test). Values are means + S.E.M.

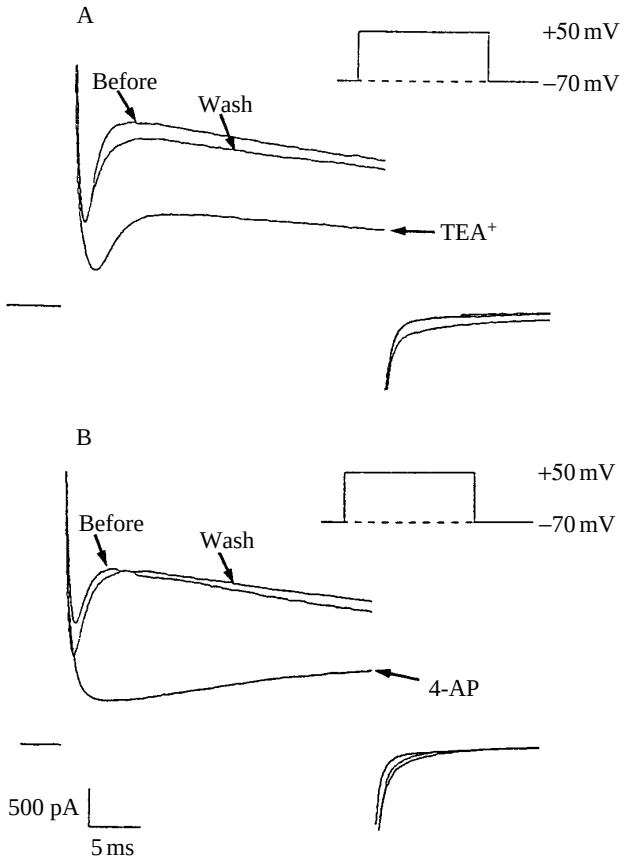

Fig. 4. Single depolarizing voltage steps from −70 to +50 mV show the effects of tetraethylammonium (TEA^+, 30 mmol l^{-1}) and 4-aminopyridine (4-AP, 5 mmol l^{-1}) on macroscopic K^+ currents recorded in the presence of TTX (0.01 μmol l^{-1}) and $CdCl_2$ (500 μmol l^{-1}). (A) TEA^+ reduced the magnitude of both a fast, transient component and a slower-activating component of the K^+ current. (B) The effects of 4-AP are more selective. The magnitude of a fast, transient component was reduced significantly, revealing the slower activation rate of the sustained component.

magnitude of the 4-AP-sensitive K^+ current, current profiles recorded in the presence of 4-AP (Fig. 5B) were subtracted electronically from those elicited in the same cells prior to 4-AP application (Fig. 5A). The resulting difference currents are shown in Fig. 5C. An *I–V* plot of current maxima from these 4-AP-sensitive currents shows the activation threshold for the fast, transient, A-type component (I_A) to be around −30 mV (Fig. 6A,C). Activation of the slower component (I_{KV}), from measurements of peak current recorded in the presence of 4-AP (Fig. 5B), occurs at a more depolarized potential, around −10 mV (Fig. 6B,C). The two currents could also be dissociated by steady-state voltage inactivation. The fast, transient outward current, but not the slowly activating outward current, could be abolished by setting the holding potential to −40 mV (Fig. 7A,B). Upon resumption of the normal holding potential of −70 mV, the transient current returned (Fig. 7C). Thus, the behaviour of the transient outward current is reminiscent of that of an A-type current.

4-AP difference currents (Fig. 5C) were used to examine the kinetics of the A-type current. The properties of the slower-activating current were examined using current profiles recorded in the presence of 4-AP (Fig. 5B). The time-to-peak of both types of K^+ current is voltage-dependent, decreasing as the size of the depolarizing voltage step increases (Fig. 8A,B). With a step from −70 to +50 mV, the fast-activating current reaches its peak level in approximately 5 ms (Fig. 8A,C), compared with a time-to-peak of 50 ms for the slower current (Fig. 8B,C). Unlike the delayed current, the A-type current inactivates during the voltage step (see Fig. 5C).

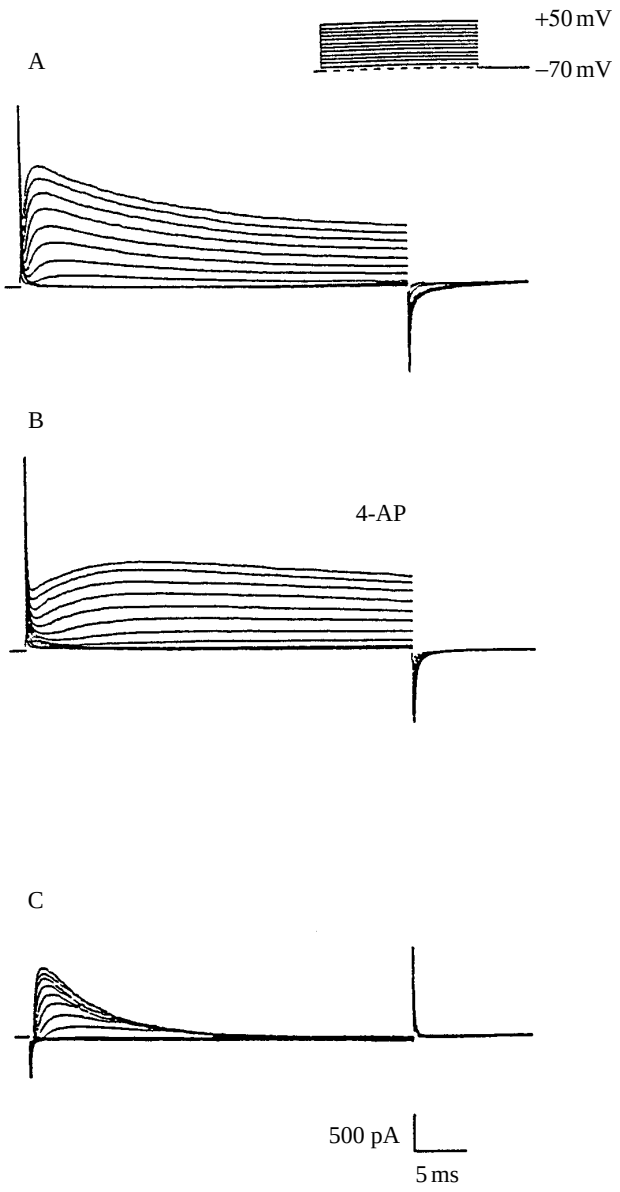

Fig. 5. Selective blockade of A-type K^+ current by 5 mmol l^{-1} 4-AP. Depolarizing voltage steps of 150 ms duration were used to examine the magnitude and time course of K^+ current blocked by 4-AP. (A) Whole-cell current profiles measured before exposure to 4-AP. (B) Blockade of a fast, transient component reveals a slower-activating K^+ current. (C) Difference currents show the current blocked by 4-AP. 4-AP is an effective blocker of A-type current in these cells, leaving a slower-activating K^+ current largely intact. Outward currents were recorded in the presence of TTX (0.01 μmol l^{-1}) and $CdCl_2$ (500 μmol l^{-1}).

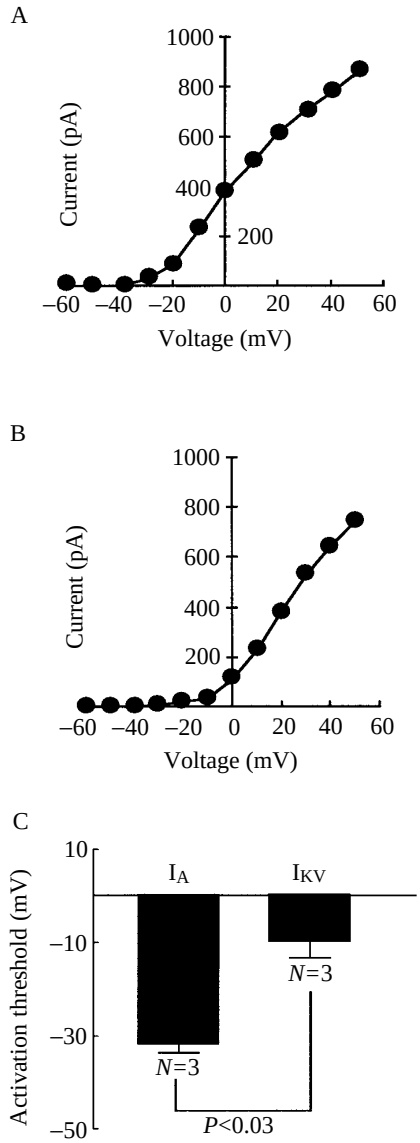

Fig. 6. Comparison of current–voltage (*I–V*) relationships for fast and slow components of the macroscopic K^+ current recorded in the presence of TTX (0.01 μmol l^{-1}) and $CdCl_2$ (500 μmol l^{-1}). (A) *I–V* plot of 4-AP difference currents shown in Fig. 4C. (B) *I–V* plot of K^+ currents remaining after blockade of fast, transient current by 4-AP (see Fig. 4B). (C) A between-group analysis indicates that the threshold for activation of the delayed current is significantly more depolarized than that of the A-type K^+ current in these cells (P<0.03 using one-tailed *t*-test). Values are means + S.E.M.

For voltage steps between +20 and +50 mV, the time constant for inactivation of this current is relatively constant (Fig. 8D).

Effects of 5-HT on K^+ currents

5-HT-modulation of a fast, transient component of the macroscopic K^+ current is clearly apparent from 5-HT difference currents shown in Fig. 2. 5-HT reduced the magnitude of this current. The 5-HT difference currents indicate that a slower-activating component was also modulated by 5-HT. This was confirmed by blocking the fast, transient current with 4-AP and examining the effects of 5-HT on the slower-activating current in isolation (Fig. 9); 5-HT reduced the magnitude of this current (Fig. 9C,D) and failed to cause a significant increase in the time-to-peak (Fig. 8C). The effect of 5-HT on the transient current was determined by subtracting the current in the presence of 5-HT from the current in the absence of 5-HT, measured with (e.g.

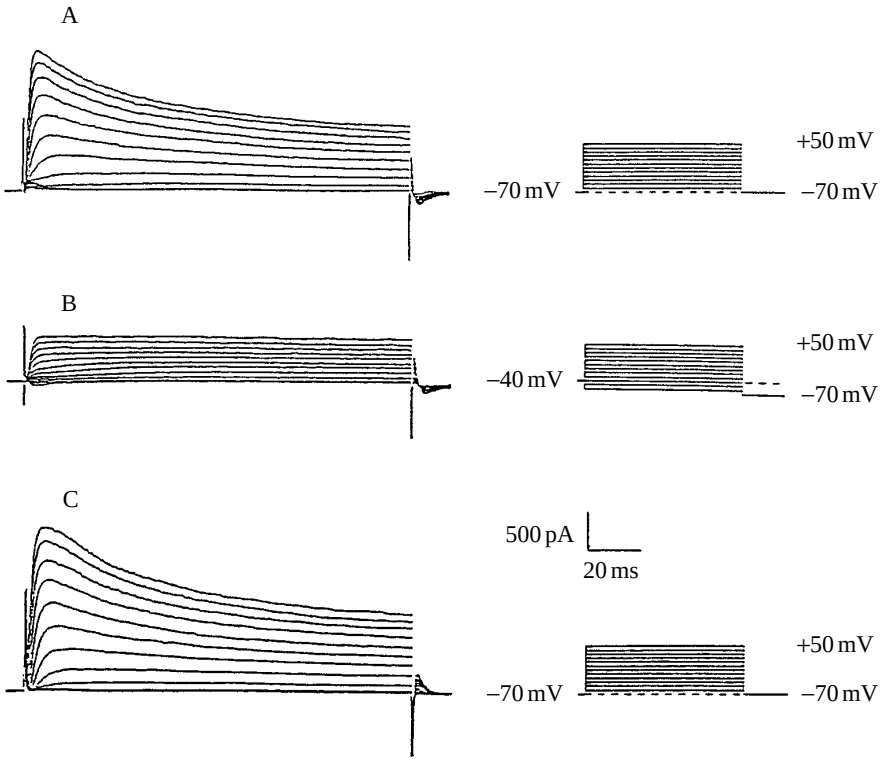

Fig. 7. Voltage inactivation of A-type K^+ current. (A) Outward current elicited by a series of depolarizing voltage steps from a holding potential of −70 mV. (B) Inactivation of the fast, transient (A-type) current using a holding potential of −40 mV. (C) Return of A-type K^+ current upon resumption of the normal holding potential of −70 mV.

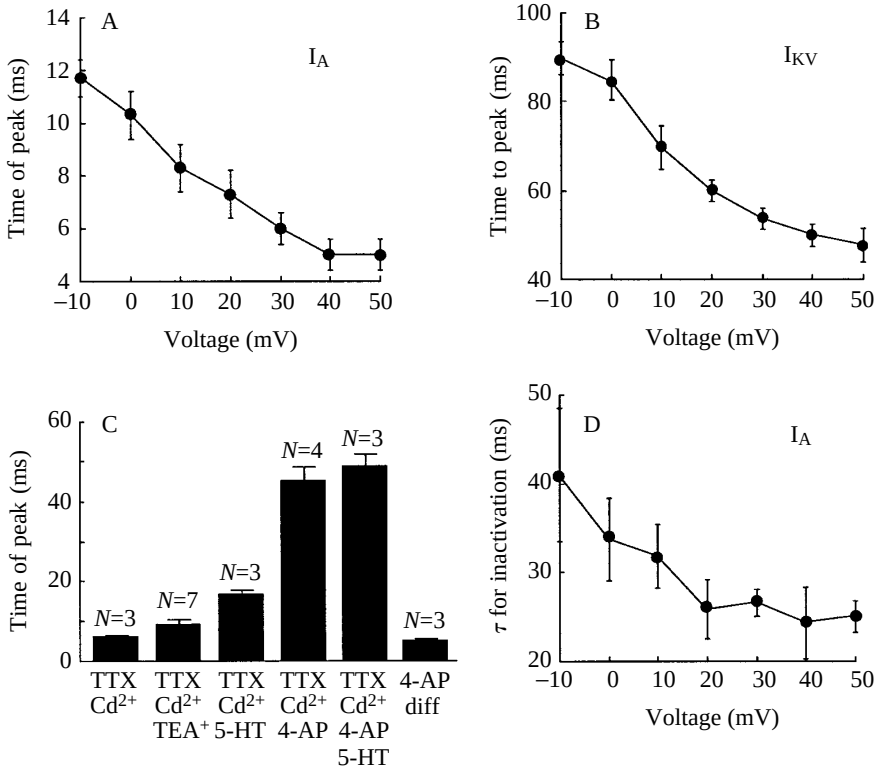

Fig. 8. Comparison of fast and slow K^+ currents. (A) Time to peak of the fast, transient (A-type) current calculated from 4-AP difference currents such as those shown in Fig. 5C (N=3). (B) Time to peak of delayed currents (N=3) recorded in the presence of 4-AP (e.g. Fig. 5B). (C) Comparison of the effects of blocking agents used in this study on the time to reach peak current levels with single steps from −70 to +50 mV. 4-AP diff, measurements from 4-AP difference currents (e.g. Fig. 5C). (D) Time constants (τ) for inactivation of the fast, transient current measured from 4-AP difference currents such as those shown in Fig. 5C (N=3). Error bars represent ± S.E.M.

Fig. 9D) and without (e.g. Fig. 9B) 4-AP. The effect of 5-HT on the fast, transient current did not differ significantly from the effect of this amine on the slower-activating current (Fig. 10). The effects of 5-HT on both types of K^+ currents were reversible.

Isolation of calcium currents

To isolate voltage-activated Ca^{2+} currents in the cells, K^+ currents were blocked by replacing K^+ with Cs^+ in the patch pipette and adding TEA^+ (30 mmol l^{-1}) to the solution bathing the cells (see Materials and methods). Blockade of outward

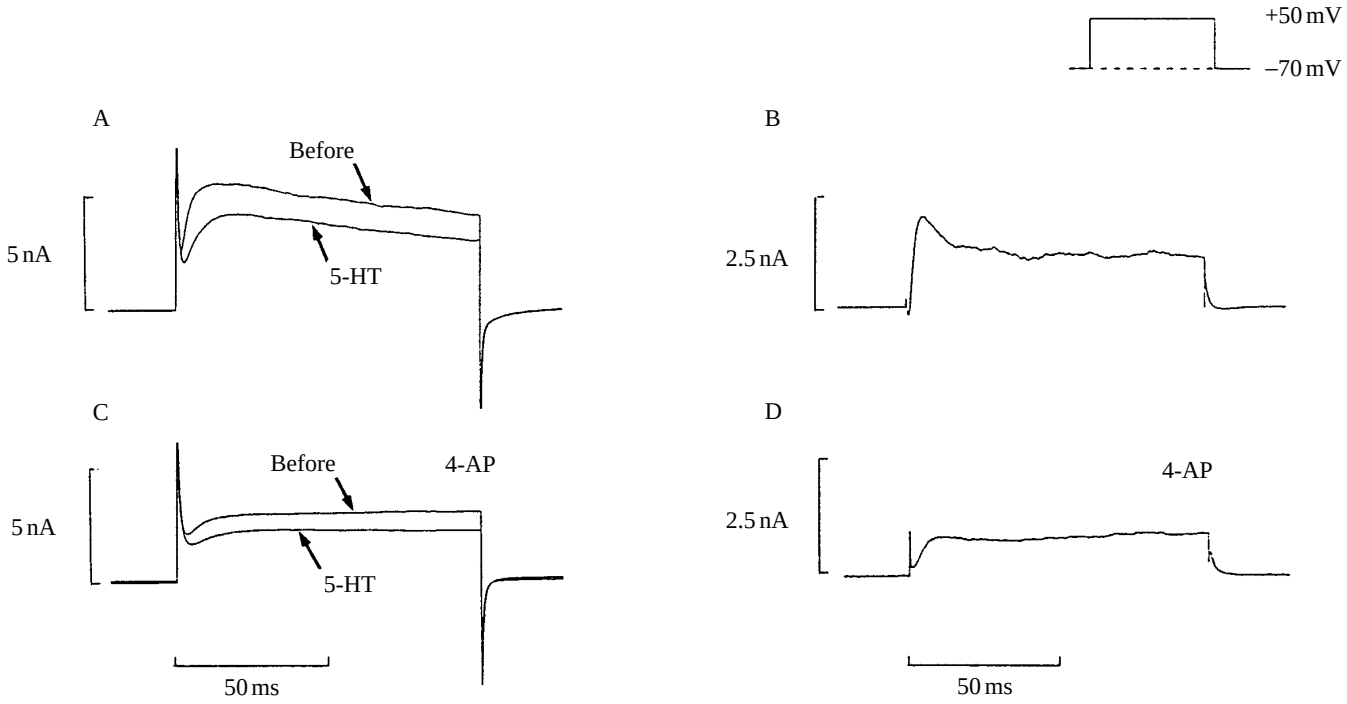

Fig. 9. 5-HT-modulation of K^+ currents. (A) Single steps from −70 to +50 mV show the effects of 5-HT (50 μmol l^{-1}) on K^+ currents prior to blockade of the fast component with 4-AP (5 mmol l^{-1}). (B) Subtraction of current recorded in the presence of 5-HT from that measured before 5-HT application (see A) provides an indication of the current blocked by 5-HT. 5-HT modulation of the A-type current is clearly apparent. (C) Blockade of the A-type current with 4-AP to show 5-HT modulation of the delayed K^+ current in isolation. (D) Subtraction of voltage-activated current recorded in the presence of 5-HT from that obtained prior to 5-HT application when 4-AP is present (see C) shows the effects of 5-HT on the delayed K^+ current in isolation. Outward currents were recorded in the presence of TTX (0.01 μmol l^{-1}) and $CdCl_2$ (500 μmol l^{-1}).

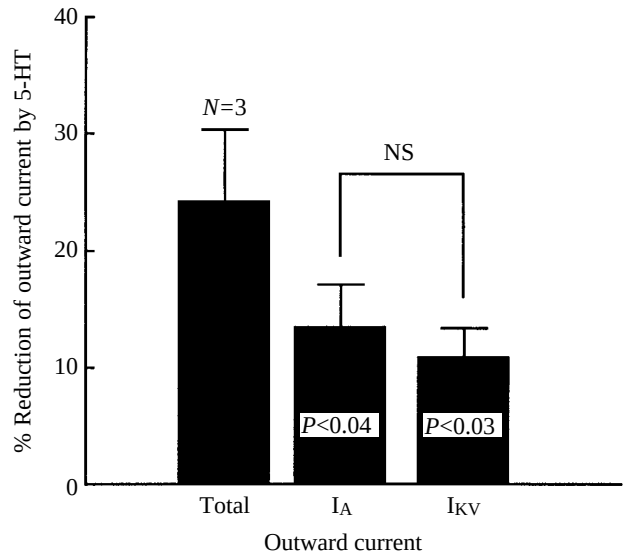

Fig. 10. Comparison of percentage reduction by 5-HT (50 μmol l^{-1}) of A-type (I_A) and delayed (I_{KV}) K^+ current. 5-HT reduced significantly the A-type (P<0.04 using one-tailed t-test) and the delayed K^+ current (P<0.03 using one-tailed t-test). A between-group comparison revealed no significant difference (NS) between the percentage reduction of these two K^+ currents by 50 μmol l^{-1} 5-HT. Values are means + S.E.M.

currents revealed a sustained inward current activated by depolarizing voltage steps applied to the cells (Fig. 11A). The time-to-peak of the inward current was voltage-dependent (Fig. 11B), with an activation threshold of −37±1.8 mV (N=8) (Fig. 11C). Steady-state inactivation of the current was voltage-dependent (Fig. 11C). As in other *M. sexta* neurones (Hayashi *et al.* 1992; Hayashi and Levine, 1992), the magnitude of inward current could be enhanced by replacing Ca^{2+} with Ba^{2+} in the saline solution bathing the cells (not shown) or blocked with the Ca^{2+} channel blocker $CdCl_2$ (500 μmol l^{-1}). Taken together, these results suggest that the inward current observed in these cells is carried by Ca^{2+}.

5-HT modulation of Ca^{2+} currents

5-HT significantly reduced the magnitude of Ca^{2+} currents in cells taken from stage 5 pupae (Fig. 12A) and increased the time taken to reach maximum current levels (Fig. 12B). Comparison of data from cells treated with 50 or 500 μmol l^{-1} 5-HT suggests that the effects of 5-HT on Ca^{2+} current are dose-dependent. Blockade of Ca^{2+} current by a high concentration of 5-HT (500 μmol l^{-1}) reveals a TEA^+-insensitive outward current in some cells (Fig. 13), the identity of which has yet to be determined. Preliminary evidence suggests that Ca^{2+} currents in RR neurones from animals at later stages of metamorphic adult development (stages 9–15) are less susceptible to modulation by 5-HT.

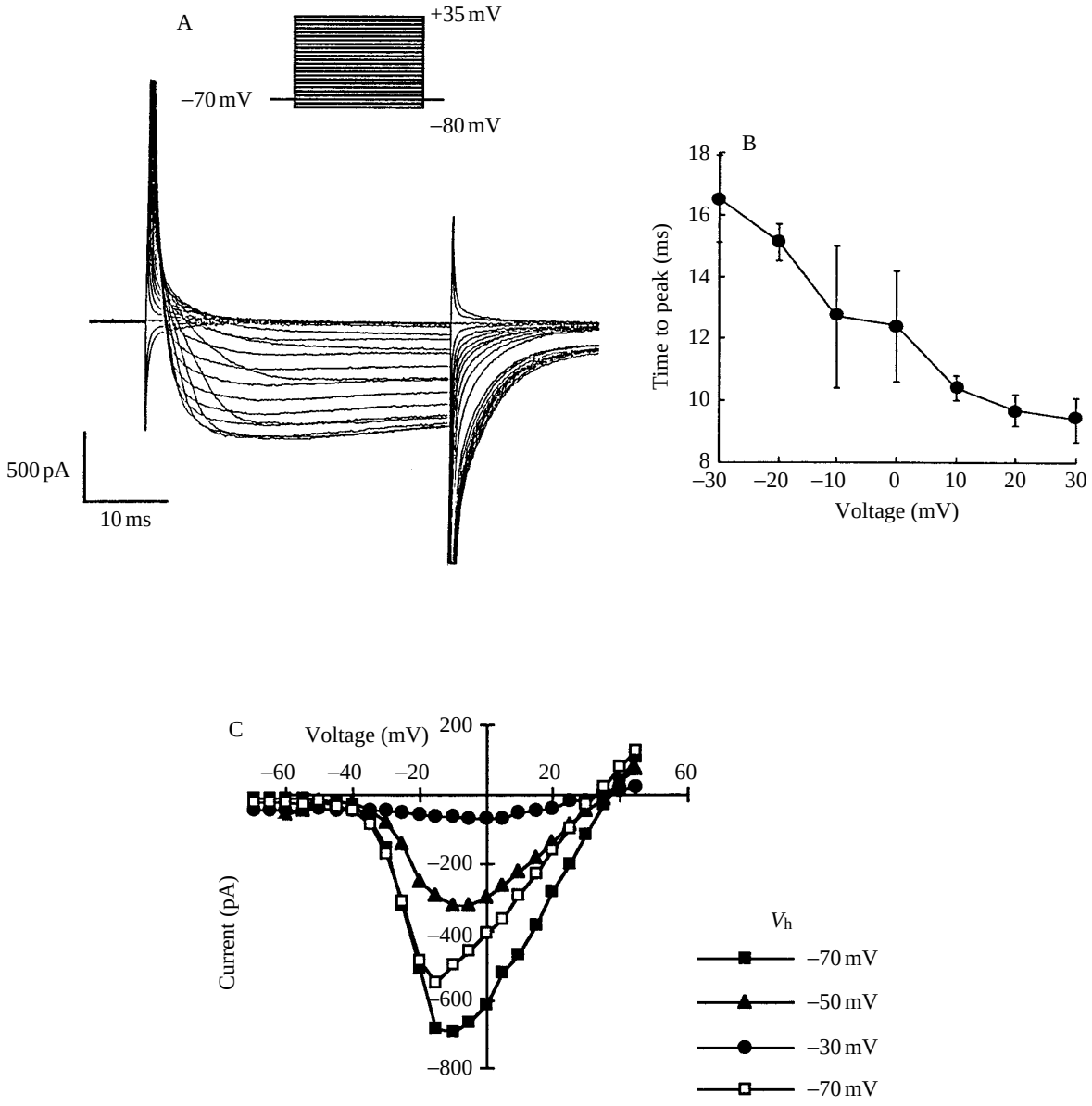

Fig. 11. Identification of Ca^{2+} currents. (A) Sustained inward current measured in the cells after blockade of outward current (see Materials and methods). (B) Time to peak current is voltage-dependent. Error bars represent ± S.E.M.; N=8. (C) Current–voltage relationships showing peak inward current at different holding potentials. The holding potentials (V_h) tested are indicated in the key. Ca^{2+} currents activated around −40 mV. Steady-state inactivation is voltage-dependent.

Discussion

We have presented evidence that pulse application of 5-HT inhibits outward A-type and delayed-rectifier-type K^+ currents, as well as voltage-activated Ca^{2+} current, in a morphologically identifiable subtype of *M. sexta* central olfactory neurones in culture. The observed modulatory effects of 5-HT on K^+ currents are consistent with the increases in excitability of mature AL neurones that result from bath application of 5-HT to the ALs of the adult moth, measured *in situ* using intracellular recording techniques (Kloppenburg and Hildebrand, 1995). Our findings suggest that reduction of outward current and the resulting loss of rectifying ability of the cells underlie the observed increases in cell excitability induced by 5-HT. 5-HT does not appear to modulate all central olfactory neurones in this way, however. Several subtypes of AL neurones in our cultures showed no response to pulse application of 5-HT, suggesting that only certain neurones from stage 5 ALs are sensitive to short-term modulation by this amine. This also appears to be the case for neurones in the ALs of the adult moth (Kloppenburg and Hildebrand, 1995).

The rationale for studying cultured neurones derived from stage 5 pupae, as opposed to pharate adults (stage 18), is based on our intention to trace the effects of 5-HT on neurones throughout their development. It is known that 5-HT-IRNs in

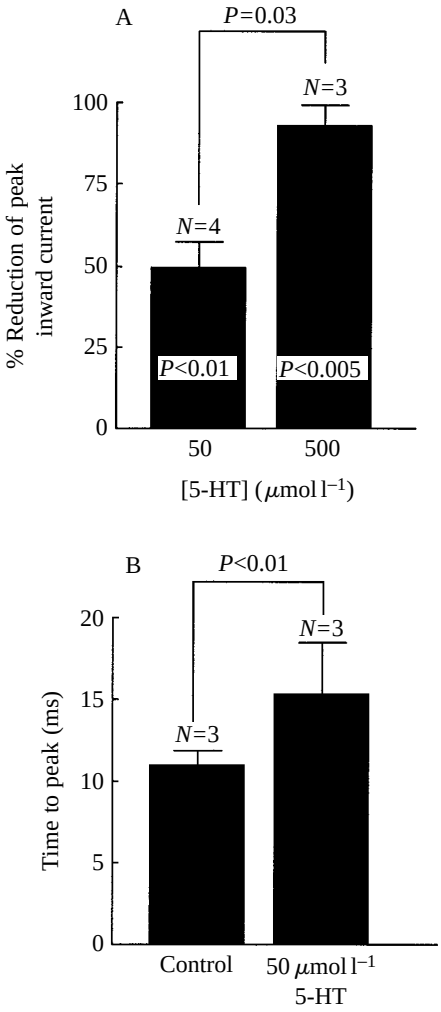

Fig. 12. (A) Percentage reduction of peak inward current by 5-HT. Inward current is reduced significantly by 50 μmol l^{-1} 5-HT (P<0.01 using two-tailed t-test) and by 500 μmol l^{-1} 5-HT (P<0.005 using two-tailed t-test). Effects of 5-HT on this current are dose-dependent. A between-group analysis reveals a significant difference between the percentage reduction caused by 50 μmol l^{-1} and 500 μmol l^{-1} 5-HT (P=0.03 using one-tailed t-test). (B) Effect of 5-HT on time taken to reach peak current levels following single voltage steps from −70 to −10 mV. A between-group analysis shows that the time to reach peak current levels is significantly greater in the presence of 5-HT (P<0.01 using two-tailed t-test).

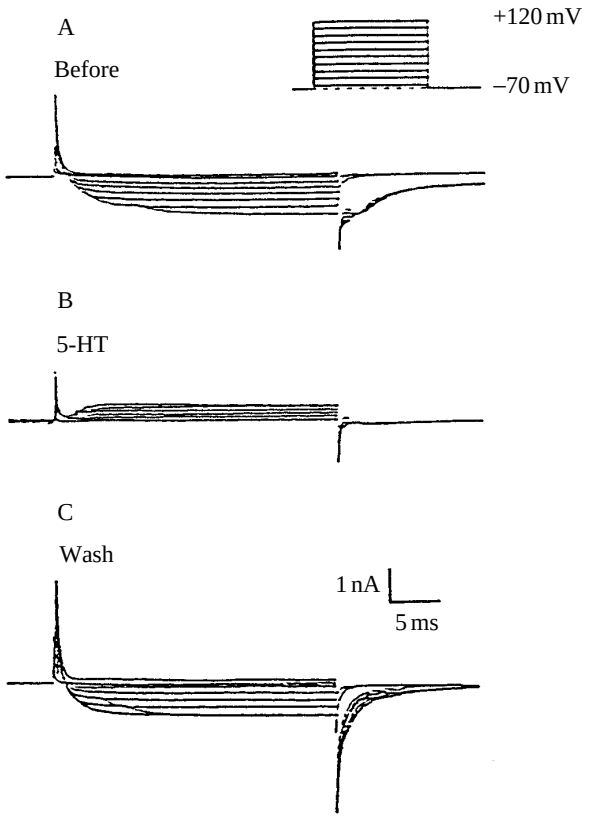

Fig. 13. Modulation of Ca^{2+} current by 5-HT. Reversible blockade of Ca^{2+} current resulting from application of 5-HT (500 μmol l^{-1}). Ca^{2+} currents were measured in the presence of TEA^+ (30 mmol l^{-1}). Blockade of inward currents reveals a TEA^+-insensitive outward current in the cells (see B).

the brain of *M. sexta* are born early and persist throughout postembryonic development (Kent *et al.* 1987; Granger *et al.* 1989; Radwan *et al.* 1989) and that, despite the extensive growth and reorganization of the AL that accompanies metamorphosis (Tolbert *et al.* 1983; Hildebrand, 1985; Tolbert, 1989; Tolbert and Oland, 1989), the single 5-HT-IRN that projects to each AL remains closely associated with the AL neuropile throughout development (Kent *et al.* 1987). Although we are unable at present to assess the effects of 5-HT on neuronal development, our finding that 5-HT can modulate outward currents in some AL neurones appears to be relevant to the behaviour of AL neurones in the brain of the adult moth. Work in progress addresses the effects of 5-HT on neurones derived from *M. sexta* at more advanced stages of metamorphic adult development.

In addition to its effects on outward K^+ currents, pulse application of 5-HT also inhibits inward Ca^{2+} current in RR neurones from stage 5 *M. sexta* pupae. The conductance underlying this current is characterized by an activation voltage around −40 mV and relatively slow inactivation. The voltage-sensitivity of the current is similar to that reported for Ca^{2+} currents identified in other insect systems (Byerly and Leung, 1988; Pearson *et al.* 1993), including leg motor neurones of *M. sexta* (Hayashi and Levine, 1992). Transient Ca^{2+} current, similar to that described by Pearson *et al.* (1993) in locust neurones, was not observed in stage 5 RR neurones. 5-HT modulation of Ca^{2+} current in *M. sexta* AL neurones may have developmental implications. A body of evidence suggests that voltage-dependent Ca^{2+} influx could regulate the differentiation of neurones in culture (Walicke and Patterson, 1981; Bixby and Spitzer, 1984; Cohan *et al.* 1987; Vidal *et al.* 1989; Holliday and Spitzer, 1990; Holliday *et al.* 1991), and there is growing evidence, in both vertebrates and invertebrates, that 5-HT can function as a developmental signal in the central nervous system (Lauder and Krebs, 1978; Haydon *et al.* 1984, 1987; McCobb *et al.* 1988*a*,*b*; Goldberg

and Kater, 1989; Lipton and Kater, 1989; Glanzman *et al.* 1990; Goldberg *et al.* 1990; Lauder, 1990). We are beginning to study the effects of chronic application of 5-HT on the morphology and growth rate of *M. sexta* AL neurones in primary cell culture.

Two major types of K^+ currents have been identified in cultured *M. sexta* AL neurones: a fast, transient current susceptible to blockade with 4-AP, and a slower-activating current blocked by TEA^+. Fast, transient (A-type) channels that activate rapidly in response to depolarizing voltage steps and inactivate within tens or hundreds of milliseconds with maintained depolarization are expressed by most excitable cells (see Rogawski, 1985; Salkoff *et al.* 1992), with the notable exception of the squid giant axon (Hodgkin and Huxley, 1952). A-type K^+ channels have been observed in a number of types of insect cells (e.g. Salkoff and Wyman, 1983; Solc and Aldrich, 1988; Laurent, 1991; Saito and Wu, 1991; Hayashi *et al.* 1992; Hayashi and Levine, 1992). In neurones of *Drosophila melanogaster* these fast, transient currents have been implicated in the regulation of action potential initiation, membrane repolarization and repetitive firing of the cells (Saito and Wu, 1991), and inhibition of A-type current by 4-AP can prolong neurotransmitter release at axon terminals (Jan *et al.* 1977; Ganetsky and Wu, 1982; Saito and Wu, 1991). Mutations at the *shaker* locus that alter A-type current in *D. melanogaster* muscle cells (Salkoff and Wyman, 1981, 1983) also broaden the action potentials in cervical giant fibres of this insect (Tanouye *et al.* 1981, 1986). Delayed K^+ currents that activate at potentials more positive than does the A-type current and show no appreciable inactivation during a depolarizing voltage step also contribute to action potential repolarization (Saito and Wu, 1991), as do Ca^{2+}-activated K^+ currents in some cells (e.g. Blatz and Magleby, 1987; Latorre *et al.* 1989). The effects of blockade of delayed K^+ currents in *M. sexta* AL neurones by TEA^+ are consistent with the pharmacology of other delayed-rectifier subtypes of K^+ channels (see Hille, 1992). We cannot rule out the possibility, which has yet to be investigated, that Ca^{2+}-dependent K^+ currents may also be present in *M. sexta* AL neurones.

The involvement of K^+ channels in setting resting membrane potentials, shaping action potential waveforms and modulating the frequency of neuronal firing has been well established (see Salkoff *et al.* 1992). In sensory neurones of the marine mollusc *Aplysia californica*, 5-HT has been found to modulate the activity of several K^+ conductances, including a relatively voltage-independent membrane channel, the S-channel, which contributes to the resting potential of the neurones (Klein and Kandel, 1980; Klein *et al.* 1982; Pollock *et al.* 1985; Shuster *et al.* 1985; Siegelbaum *et al.* 1982), a voltage-dependent current similar to the delayed K^+ current I_{kv} (Baxter and Byrne, 1989) and a steady-state Ca^{2+}-activated K^+ current also present in these cells (Boyle *et al.* 1984; Ewald and Eckert, 1983; Walsh and Byrne, 1989). 5-HT-modulation of multiple K^+ conductances is also found in the vertebrate brain. In hippocampal slices, 5-HT activates a Ca^{2+}-independent K^+ current responsible for hyperpolarization and inhibition of CA1 neurones, it suppresses a slow Ca^{2+}-dependent K^+ conductance (I_{AHP}) in these cells that is largely responsible for accommodation of cell firing, and it causes long-lasting suppression of a voltage-dependent K^+ conductance (I_m) that leads to neuronal depolarization and excitation (see Colino and Halliwell, 1987). 5-HT also affects hippocampal neurones in primary embryonic cell culture in a number of ways (Yakel *et al.* 1988), the most commonly observed of which is an increase in K^+ conductance similar to that described in slice preparations (Andrade *et al.* 1986; Colino and Halliwell, 1987). In addition to its effects on ionic conductances of cell membranes, 5-HT can also influence subcellular processes, such as Ca^{2+}-handling (Boyle *et al.* 1984) and transmitter mobilization (Gingrich *et al.* 1988; Hochner *et al.* 1986). The results of the present investigation suggest that modulation of K^+ channel activity by 5-HT may be responsible for the 5-HT-induced changes in AL neurone excitability observed in the brain of the adult moth (Kloppenburg and Hildebrand, 1995). We are currently studying the effects of 5-HT on AL neurones at early and late stages of metamorphic adult development, in order to investigate the effects of 5-HT on signal modulation and on mechanisms of neuronal development.

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