Plateau Potentials in Developing Antennal-Lobe Neurons of the Moth, *Manduca sexta*

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Plateau potentials in developing antennal-lobe neurons of the moth, *Manduca sexta*. *J Neurophysiol* 93: 1949–1958, 2005. First published November 17, 2004; doi:10.1152/jn.01050.2004. Using whole cell recordings from antennal-lobe (AL) neurons in vitro and in situ, in semi-intact brain preparations, we examined membrane properties that contribute to electrical activity exhibited by developing neurons in primary olfactory centers of the brain of the sphinx moth, *Manduca sexta*. This activity is characterized by prolonged periods of membrane depolarization that resemble plateau potentials. The presence of plateau potential–generating mechanisms was confirmed using a series of tests established earlier. Brief depolarizing current pulses could be used to trigger a plateau state. Once triggered, plateau potentials could be terminated by brief pulses of hyperpolarizing current. Both triggering and terminating of firing states were threshold phenomena, and both conditions resulted in all-or-none responses. Rebound excitation from prolonged hyperpolarizing pulses could also be used to generate plateau potentials in some cells. These neurons were found to express a hyperpolarization-activated inward current. Neither the generation nor the maintenance of plateau potentials was affected by removal of Na+ ions from the extracellular medium or by blockade of Na+ currents with TTX. However, blocking of Ca2+ currents with Cd2+ (5 × 10−4 M) inhibited the generation of plateau potentials, indicating that, in *Manduca* AL neurons, plateau potentials depend on Ca2+. Examining Ca2+ currents in isolation revealed that activation of these currents occurs in the absence of experimentally applied depolarizing stimuli. Our results suggest that this activity underlies the generation of plateau potentials and characteristic bursts of electrical activity in developing AL neurons of *M. sexta*.

INTRODUCTION

Developing neurons in primary olfactory centers [antennal lobes (ALs)] of the brain of the sphinx moth, *Manduca sexta*, show bursts of electrical activity (Mercer and Hildebrand 2002a; Oland et al. 1996). Here, we examine intrinsic properties that contribute to the characteristic form of electrical activity apparent in these developing neurons: their ability to generate prolonged membrane depolarizations or plateau potentials.

In holometabolous insects such as moths, the architecture of the ALs changes dramatically during metamorphic development. *Manduca* larvae hatch from eggs and pass through five larval instars before undergoing metamorphosis from larva to pupa to adult. Pupal development can be divided into 18 stages, each of which lasts ~1 day (Sanes and Hildebrand 1976a,b; Tolbert et al. 1983). At the onset of pupal development (pupal stages 1 and 2; Sanes and Hildebrand 1976a,b), olfactory receptor cells are born in the antennal epithelium. These primary sensory afferent neurons extend axons toward the ALs of the brain, where they trigger the formation, late in pupal stage 3, of subunits of synaptic neuropil called glomeruli (Hildebrand 1985; Tolbert et al. 1983). The glomeruli develop in a lateral-to-medial wave that crosses the AL neuropil (Malun et al. 1994). Each glomerulus is invaded by local interneurons, the processes of which are restricted to the AL neuropil, and by AL projection (output) neurons, which transfer information from the AL to higher-order centers, such as the mushroom bodies of the brain. Centrifugal neurons contribute also to the complex, highly structured glomerular neuropil of the ALs. A large serotonin-immunoreactive neuron enters the developing AL around pupal stage 6 and sends a dense tuft of arbors into each glomerulus (Kent et al. 1987; Oland et al. 1995). Previous reports have shown that serotonin (5HT) affects the growth (Mercer et al. 1996a) as well as the excitability (Kloppenburg and Heinbockel 2000; Kloppenburg and Hildebrand 1995; Kloppenburg et al. 1999; Mercer et al. 1995, 1996b) of *Manduca* AL neurons.

Rapid changes in AL morphology coincide temporally with changes in the electrophysiological properties and response characteristics of AL neurons (Mercer and Hildebrand 2002a,b). Action potentials in neurons from *Manduca* ALs early in metamorphosis are generally small in amplitude, long in duration, and Ca2+-dependent, but as development proceeds, they become larger in amplitude, shorter in duration, and increasingly Na+-dependent. Developmental changes in voltage-gated and Ca2+-dependent ionic currents contribute to the emergence of cell type–specific response characteristics in the cells (Mercer and Hildebrand 2002a,b).

During metamorphosis, including critical stages of glomerulus formation, electrical activity can be detected in antennal nerve (sensory afferent) fibers and in AL neurons of the moth (Mercer and Hildebrand 2002a; Oland et al. 1996). In AL neurons, this activity is characterized by prolonged membrane depolarizations that resemble plateau potentials (Mercer and Hildebrand 2002a). Here we confirm the presence of plateau potential–generating mechanisms in *Manduca* AL neurons and show that the formation and maintenance of plateau potentials in developing AL neurons of the moth is Ca2+-dependent.

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METHODS

Manduca sexta (Lepidoptera: Sphingidae)

Animals were reared on an artificial diet (modified from that of Bell and Joachim 1976) and maintained at 25°C and 50–60% relative humidity under a long-day photoperiod regimen (17-h light/7-h dark). AL neurons were either dispersed and maintained in culture or examined in situ using semi-intact brain preparations. The majority of cells examined in this study were from pupae at developmental stages 6–12 (n = 86), but small numbers of cells were also examined from early (stage 4; n = 5) and late (stages 14–16, n = 4) stages of metamorphosis.

AL neurons in vitro

Cells were maintained in vitro according to methods described previously by Hayashi and Hildebrand (1990). Brains removed from cold-anesthetized pupae were placed into sterile culture saline containing (in mM) 149.9 NaCl, 3 KCl, 3 CaCl₂, 0.5 MgCl₂, 10 TES [N-tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid], and 11 5-glucose and 6.5 g/l lactalbumin hydrolysate (GIBCO), 5 g/l TC Yeastolate (DIFCO), 10% fetal bovine serum (FBS, Hyclone), 100 mAimidazole, 100 mA350 mg glucose, 335 mg malic acid, 30 mg succinic acid, 1.4 g TC 26°C, and the cells were maintained for a minimum of 5 days and a metamorphosis.

neuronal somata. To aid the removal of glial cells that envelop the neuron somata, the brain was placed for 3–5 min in enzyme (0.5 mg/ml collagenase and 2 mg/ml Dispase). The preparation was rinsed thoroughly with insect saline solution (see Electrophysiological recording) and mounted with fine pins in a dish lined with Sylgard (Dow Corning).

Electrophysiological recording

Patch-clamp recordings in whole cell configuration (Hamill et al. 1981) were used to examine the electrical properties of AL neurons. Electrod with resistances of 1–2 MΩ were made from borosilicate glass (100-μl micropipettes, 1.71 mm OD, 1.32 mm ID; VWR Scientific, West Chester, CA) and filled with a solution containing (in mM) 150 K-aspartate, 5 NaCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, and 10 HEPES (pH 7), and adjusted to 330 mOsM with mannitol. Cells were viewed with an IMT-2 inverted microscope (Olympus) equipped with Hoffman modulation contrast optics. To facilitate the formation of high-resistance (gigaohm) seals, culture medium was replaced with insect saline solution containing (in mM) 100 NaCl, 4 KCl, 6 CaCl₂, 5 5-glucose, and 10 HEPES (pH 7), adjusted to 360 mOsM with mannitol, prior to recording. Cells were continuously superfused with fresh saline solution throughout the recording period, and junction potentials were nullified prior to seal formation. To obtain whole cell recordings, light suction and brief high-voltage pulses were used to rupture the cell membrane beneath the recording electrode. Recordings were made using an AxoPatch 1B amplifier (Axon Instruments, Union City, CA), and data were acquired and analyzed using pClamp 6 software (version 6.02, Axon Instruments). Membrane responses were sampled at intervals of 100 μs and were filtered at 2 kHz with a low-pass 4-pole Bessel filter. Linear leakage currents were subtracted on-line from all records. Electrical activity and plateau potential properties of the cells were examined under current clamp. Recordings under voltage clamp were used to identify currents underlyng the generation of plateau potentials in the cells.

Identification of plateau potential mechanisms

The following tests described by Russell and Hartline (1982) were used to confirm the presence of plateau-potential mechanisms in developing Manduca AL neurons.

1) Trigger test: brief (20 ms) depolarizing current pulses (0.05–2 nA) were used to identify cells in which it was possible to trigger plateau potentials.

2) Termination test: in cells in which a plateau state could be generated, brief (20 ms) hyperpolarizing current pulses (ca. –0.5 nA) were used to terminate the plateau; to clearly show that termination was induced by injection of hyperpolarizing current, the timing of the hyperpolarizing pulse in successive episodes was shifted progressively closer to the triggering pulse.

3) Threshold test: the pulses used to trigger (0.05–2 nA) or to terminate (~0.05 to –1.0 nA) the plateau state were varied in amplitude systematically to determine whether the triggering and terminating of firing states were threshold phenomena.

4) All-or-none test: stimulus intensity was also varied systematically to determine whether responses were “all-or-none” or graded with stimulus intensity.

5) Symmetrical pulse test: responses to brief (20 ms) symmetrical positive and negative current pulses were compared with examine whether positive (depolarizing) pulses would produce a greater response than negative (hyperpolarizing) pulses in cells exhibiting plateau properties.

Pharmacological analysis of plateau potentials

Routine pharmacological techniques were used to examine the contribution of ionic currents to the generation and maintenance of plateau potentials in the cells. Na⁺ currents were blocked with TTX (10⁻⁷ M) and Ca²⁺ currents with 5 × 10⁻⁴ M CdCl₂. In Na⁺-free
solutions, NaCl was replaced with Tris-Cl, and in Ca²⁺-free solutions, CaCl₂ was replaced with BaCl₂. K⁺ currents were blocked by adding 3 × 10⁻² M TEA to the solution bathing the cells. Effects on plateau potential formation of bath application of 5HT (50 μM), which reduces K⁺ current amplitudes in the cells (Kloppenburg et al. 1999; Mercer et al. 1995,1996b), were also examined in this study.

Data analysis

Effects of drug treatment on plateau potential properties (amplitude, duration, and membrane repolarization rate) were examined by comparing measurements taken prior to drug treatment (control) with measurements from the same cells 2–5 min after drug application. For pairwise comparisons, two-tailed Student’s t-test were used to assess statistical significance. A significance level of 0.05 was accepted for all tests. Data are presented as means ± SD.

RESULTS

Plateau potential properties

Prolonged depolarizations resembling plateau potentials were observed in AL neurons in vitro (Fig. 1A) and in situ, in semi-intact brain preparations (e.g., Fig. 8B). The following results confirm the presence of plateau potential–generating mechanisms in these neurons.

AL neurons in vitro exhibited prolonged depolarizations in response to brief (20 ms) pulses of depolarizing current (Fig. 2A). The responses showed signs of regenerative origin, growing after the end of the stimulus and outlasting the stimulus by significant amounts of time. Plateau potential properties recorded from cells in vitro at stages 6 to 8 of metamorphosis (n = 15) are shown in Table 1A. The plateau potential properties of stage-10 cells in situ (n = 4) are included for comparison (Table 1B). For cells in vitro, plateau potential amplitudes ranged from 8 to 29 mV (16 ± 7.3 mV). The duration of the plateau varied considerably, ranging from ~400 ms to several seconds, and was longest in cells at early stages of development. The rate of repolarization of the cell membrane after termination of the plateau was typically slow (<0.03 mV/ms, Table 1A). While similar properties were observed in cells in situ (Table 1B), the plateau potentials in these more mature (stage-10) neurons were generally shorter in duration (however, see Fig. 8). Triggering of the plateau state was a threshold phenomenon, with subthreshold pulses producing no change in state (Fig. 2B). Above threshold, however, the depolarizing level of the plateau was independent of stimulus strength (Fig. 2C).

Consistent with the presence of plateau mechanisms, symmetrical depolarizing and hyperpolarizing pulses of short-duration (20 ms) produced asymmetrical responses in Manduca AL neurons, with depolarizing current pulses producing greater responses (Fig. 3A). Once triggered, the plateau state could be terminated by brief (20 ms) hyperpolarizing current pulses (Fig. 3B). Plateau-potential termination was also a threshold phenomenon, because subthreshold pulses produced no change in state (Fig. 3C).

In some but not all AL neurons, rebound excitation from prolonged hyperpolarizing current pulses could also be used to generate plateau potentials. In cells in which a depolarizing sag toward the resting membrane potential occurred during the hyperpolarizing current pulse, rebound excitation was observed at current termination (Fig. 4A, bottom trace). Recordings from 23 cells under voltage clamp revealed that, in 83% of the cells, hyperpolarizing voltage steps to membrane potentials negative to approximately –80 mV induced a slowly activating inward current that showed no inactivation during the 1-s voltage step (Fig. 4B). This current could be blocked by bath application of 1–2 mM Cs⁺ (Fig. 4, B and C). Reducing the amplitude of this current with Cs⁺ inhibited the formation of plateau potentials using prolonged hyperpolarizing current pulses.

Ionic currents involved in the generation and maintenance of plateau potentials

NA⁺ CURRENTS. Bathing cells in Na⁺-free saline (Fig. 5A), or blocking Na⁺ channels with TTX (Fig. 5B), hyperpolarized the membrane, reducing the likelihood of triggering a plateau poten-
However, if depolarizing current was used to return the membrane potential to the level recorded prior to the application of Na\(^+/\)H\(^{+}\)-free saline or TTX (e.g., Fig. 5B), plateau potentials again could be reliably generated. While neither the formation nor the maintenance of plateau potentials was affected by removal of Na\(^+/\)H\(^{+}\) ions or blocking Na\(^+/\)H\(^{+}\) channels with TTX, the amplitude of spikes riding atop the plateau was reduced.

**CA\(^{2+}\) CURRENTS.** Blocking Ca\(^{2+}\) currents in the cells with 500 \(\mu\)M CdCl\(_2\) completely inhibited the formation of plateau potentials (Fig. 5, Ci and Cii). The effects of CdCl\(_2\) could occasionally be reversed by washing cells in Cd\(^{2+}\)-free saline (Fig. 5, Ciii and Civ). To examine whether influx of Ca\(^{2+}\) ions is also required for the termination of plateau potentials, Ca\(^{2+}\) in the medium bathing the cells was replaced with Ba\(^{2+}\). In Ba\(^{2+}\), the generation of regular oscillations of the membrane potential during the plateau phase was inhibited and the membrane became more depolarized than normal (Fig. 6A). This suggested that Ca\(^{2+}\)-activated K\(^{+}\) currents regulate the level of depolarization and contribute to spike activity observed in cells in the plateau state. As plateau amplitudes were increased with

![FIG. 2. Tests used to confirm the presence of plateau potential–generating mechanisms in Manduca AL neurons. A: trigger test on a pupal stage-10 AL neuron maintained 7 days in vitro. A brief depolarizing current pulse (20 ms, 1 nA) triggers a plateau state. Response grows after the end of stimulus and outlasts stimulus by a significant amount of time. B: threshold test on a pupal stage-6 AL neuron maintained 7 days in vitro. Pulses used to trigger plateau state were varied in amplitude systematically from 0.2 to 1.0 nA. Ten episodes have been superimposed to show that triggering of the firing state is a threshold phenomenon. C: all-or-none test on a pupal stage-6 AL neuron maintained 5 days in vitro. Six episodes have been superimposed to show that, above threshold, responses to depolarizing current pulses are "all-or-none" and not graded with stimulus intensity.](http://jn.physiology.org/)

![FIG. 3. A: symmetrical pulse test on a pupal stage-8 AL neuron maintained 7 days in vitro. Symmetrical positive and negative current pulses (±0.6 nA) produce asymmetrical responses, the positive (depolarizing) pulse producing a greater response. B: termination test on a pupal stage-6 AL neuron maintained 6 days in vitro. In cells in which a plateau state could be generated, brief hyperpolarizing current pulses (−1.0 nA, 20 ms) terminated the plateau. Five episodes have been superimposed to show the effects in successive episodes of shifting the timing of the hyperpolarizing pulse progressively closer to the triggering pulse. C: termination threshold test on a pupal stage-6 AL neuron maintained 6 days in vitro. Pulses (250 ms) used to terminate the plateau state were varied in amplitude systematically from −0.1 to −0.6 nA. Six episodes have been superimposed to show that termination of the firing state is a threshold phenomenon.](http://jn.physiology.org/)

**TABLE 1. Plateau-potential properties**

<table>
<thead>
<tr>
<th>Cells</th>
<th>RMP, mV</th>
<th>Plateau Potential, mV</th>
<th>Plateau Amplitude, mV</th>
<th>Plateau Duration, ms</th>
<th>*Rate of Repolarization, mV/ms</th>
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<tr>
<td>In vitro</td>
<td>−49 ± 7.4 (15)</td>
<td>−33 ± 7.5 (15)</td>
<td>16 ± 7.3 (15)</td>
<td>809 ± 348 (11)</td>
<td>0.025 ± 0.02 (11)</td>
</tr>
<tr>
<td>In situ</td>
<td>−42 ± 2.6 (4)</td>
<td>−27 ± 2.6 (4)</td>
<td>11 ± 2.5 (4)</td>
<td>192 ± 46 (4)</td>
<td>0.02 ± 0.01 (4)</td>
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</table>

Values are mean ± SD with the number of cells in parentheses. RMP, resting membrane potential. *Measured from termination of plateau to 50% repolarization to resting level.
Ba$^{2+}$ toward potentials less negative than approximately $-10$ mV, the likelihood that they would terminate was greatly increased, and the rate of membrane repolarization following termination of the plateau state was enhanced (Fig. 6Aii; Table 2A). It is likely that this was caused by increased voltage inactivation of the Ca$^{2+}$/H11001 channels.

K$^+$ CURRENTS. To examine the contribution of K$^+$ currents to these events, cells ($n = 4$) were exposed to 3×10$^{-2}$ M TEA (Fig. 6B). In the presence of TEA, membrane oscillations were blocked, and the membrane potential during the plateau state became significantly more depolarized than normal (Table 2B). In addition, plateau potential duration was reduced and membrane repolarization following plateau termination occurred at a significantly faster rate than normal (Table 2B), possibly induced by enhanced voltage inactivation of the Ca$^{2+}$/H11001 currents. In the presence of TEA, termination of the plateau potential followed by rapid repolarization of the membrane commenced at voltages of between $-12$ and $-2$ mV ($-7.25 \pm 4.6$ mV).

Ca$^{2+}$/H11001 CURRENTS IN ISOLATION. The contribution that Ca$^{2+}$/H11001 currents make to the generation and maintenance of plateau potentials was investigated further by examining Ca$^{2+}$ currents in isolation (Fig. 7A). Cells in which Na$^+$ and K$^+$ currents had been blocked (see METHODS) continued to exhibit prolonged membrane depolarization in response to brief pulses of depolarizing current (Fig. 7B; Table 3). Prior to termination of the plateau state, the membrane potential sagged toward rest before commencing rapid repolarization around a mean breakpoint voltage of $-4.9 \pm 1.07$ mV. Influxes of Ca$^{2+}$ were also recorded in these neurons in the absence of experimentally applied depolarizing current pulses (Fig. 7C). These “spontaneous” events were abolished by bath application of 500 μM CdCl$_2$ (data not shown). Blockade of Ca$^{2+}$/H11001 currents with Cd$^{2+}$/H11001 also abolished the characteristic bursts of electrical activity observed in immature Manduca AL neurons (Fig. 1B).

FIG. 4. Plateau potential formation resulting from prolonged (800 ms) hyperpolarizing current pulses. Recordings shown are from pupal stage-6 AL neurons maintained 7 days in vitro. A: responses to a series of hyperpolarizing current pulses (0.2 to $-2$ nA) recorded under current clamp. Entry into a plateau state using this form of stimulation was observed only in cells that showed a depolarizing sag toward the resting membrane potential during the hyperpolarizing pulse (bottom trace). Bii: switching to voltage clamp revealed that hyperpolarizing voltage steps greater than approximately $-80$ mV resulted in the activation of a slow inward current that showed no inactivation during the voltage step. In all recordings, the series resistance (calculated from the capacitative charging transient) was $<5$ MOhm. With maximum currents of $<500$ pA, the series resistance error should be $<3$ mV. No correction has been made for this error. Bii: bath application of Cs$^+$ reduces amplitude of current. Ci and Cii: Cs$^+$ reduces magnitude of the sag toward rest during the hyperpolarizing pulse reducing rebound excitation and inhibiting entry into the plateau state.

FIG. 5. Removing Na$^+$ from the extracellular medium (A) or blocking Na$^+$ channels with TTX (B) had no effect on the formation or maintenance of plateau potentials in the cells. Asterisk signifies that resting membrane potential has been maintained at the level recorded prior to application of TTX using current injection. C. CdCl$_2$ blocks plateau potential formation (i and ii). Effects of Cd$^{2+}$/H11001 were generally irreversible, but in a small number of cells, block of Ca$^{2+}$/H11001 currents could be reversed by washing in Cd$^{2+}$/H11001-free saline (iii and iv). Recordings shown are from pupal stage-8 (A) and stage-10 (B and C) AL neurons maintained 7 days in vitro.

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membrane repolarization following termination of the plateau state was increased, and rate of depolarization toward potentials less negative than approximately –10 mV, the membrane became more depolarized than normal (Table 2B). As the plateau oscillations of the membrane during the plateau phase was inhibited, and the extracellular medium with Ba2+ was used to block K+ currents with 3 × 10−2 M TEA. Asterisk indicates that resting membrane potential was maintained at control levels using current injection. Table 2A). As the plateau depolarized toward potentials less negative than approximately –10 mV, the likelihood that plateau potential would terminate was increased, and rate of membrane repolarization following termination of the plateau state was enhanced (arrows, A1 and AII). B: effects of blocking K+ currents with 3 × 10−2 M TEA. Asterisk indicates that resting membrane potential was maintained with current injection at a level close to that recorded prior to TEA treatment. In the presence of TEA, membrane oscillations were blocked, and membrane potential during the plateau state became significantly more depolarized than normal (Table 2B). Duration of plateau potential was markedly reduced, and membrane repolarization following termination of the plateau (arrow) occurred more rapidly than normal. Recordings shown are from pupal stage-6 (A1) and pupal stage-8 (B) AL neurons maintained in vitro for 5 and 6 days, respectively.

Developmental changes in cell excitability

At late stages of development (stages 12–18), it became increasingly difficult to elicit plateau potentials using brief pulses of depolarizing current alone (e.g., Fig. 8A1). This change in excitability seemed to be associated with the appearance in the whole cell current profile of prominent, rapidly activating, transient K+ currents (see Mercer and Hildebrand 2002a,b). We have shown elsewhere that these K+ currents can be reduced in amplitude by exposing cells to the neuromodulator 5HT (Kloppenburg et al. 1999; Mercer et al. 1995, 1996b), and we hypothesized that 5HT-induced reduction of outward currents would enhance plateau potential formation in cells at late stages of metamorphosis. To examine this possibility, 5HT (50 μM) was bath-applied for 1–5 min to cells in vitro (stages 14–16; n = 4) or in situ in semi-intact preparations (stage 12; n = 2).

5HT alone did not trigger plateau potentials (data not shown). However, in three of four cells in vitro in which brief (20–200 ms) depolarizing pulses failed to trigger plateau potentials prior to 5HT treatment (Fig. 8A1), prolonged exposure in the external medium with Ba2+ and blocking K+ currents with TEA

The effects of replacing Ca2+ in the extracellular medium with Ba2+ under voltage clamp. Ca2+ currents were isolated pharmacologically as described in METHODS. A: depolarizing voltage steps were used to activate voltage-gated Ca2+ channels in a cell examined under voltage clamp. Ca2+ currents in Manduca AL neurons activate around –40 mV and peak at approximately –10 mV (Mercer et al. 1995 2002b). B: current-clamp recording showing potentials resulting from Ca2+ influx triggered using brief (20 ms) pulses of depolarizing current. Pulses of depolarizing current greater than –0.3 nA triggered long-lasting membrane depolarizations that were remarkably stable in amplitude and duration. C: spontaneous long-lasting membrane depolarizations resulting from Ca2+ influx occurring in the absence of external stimulation. Recordings shown are from pupal stage-10 AL neurons maintained 7 days in vitro.

FIG. 6. A: effects on plateau potential formation of replacing Ca2+ in the extracellular medium with Ba2+. Under barium, the generation of regular oscillations of the membrane during the plateau phase was inhibited, and the membrane became more depolarized than normal (Table 2A). As the plateau depolarized toward potentials less negative than approximately –10 mV, the likelihood that plateau potential would terminate was increased, and rate of membrane repolarization following termination of the plateau (arrow) occurred more rapidly than normal. Recordings shown are from pupal stage-6 (A) and pupal stage-8 (B) AL neurons maintained in vitro for 5 and 6 days, respectively.

TABLE 2. Effects on plateau-potential properties of replacing Ca2+ with Barium and blocking K+ currents with TEA

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<tr>
<th></th>
<th>RMP, mV</th>
<th>Plateau Potential, mV</th>
<th>Plateau Amplitude, mV</th>
<th>Plateau Duration, ms</th>
<th>‡Rate of Repolarization, mV/ms</th>
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<tr>
<td>Control</td>
<td>−50 ± 5 (3)</td>
<td>−48 ± 2.9 (3)</td>
<td>−36 ± 10 (3)</td>
<td>−22 ± 2 (3)</td>
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<td>Barium</td>
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Replacing

<table>
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<td></td>
<td>−43 ± 2.2 (3)</td>
<td>−43 ± 2.2 (3)</td>
<td>−28 ± 2.5 (3)</td>
<td>7 † ± 2.5 (3)</td>
<td>13 ± 2.1 (3)</td>
<td>48 † ± 4.4 (3)</td>
<td>890 ± 285 (3)</td>
<td>138 ± 35 (3)</td>
<td>0.01 ± 0.01 (3)</td>
<td>0.36 † ± 0.09 (3)</td>
</tr>
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</table>

Blocking

Values are mean ± SD with number of cells in parentheses. *Resting membrane potential (RMP) retained at control levels using current injection. †Significantly different from Control. ‡Measured from termination of plateau to 50% repolarization to resting level.
sure to 5HT (>3 min) not only increased the number of action potentials elicited by depolarizing current pulses (described in detail elsewhere; Kloppenburg and Heinbockel 2000; Kloppenburg and Hildebrand 1995; Kloppenburg et al. 1999; Mercer et al. 1995, 1996b) but also promoted the cell’s entry into a plateau state (Fig. 8Aii). Cells in situ exhibiting bursts of electrical activity also responded to 5HT with increases both in the number of spikes and in the number and duration of plateau potentials recorded in the cells (Fig. 8B).

**DISCUSSION**

*Manduca AL neurons exhibit plateau potential properties*

Developing *Manduca* AL neurons generate prolonged membrane depolarizations that resemble plateau potentials. Here we show that this is an intrinsic property and that it depends on the influx of Ca\(^{2+}\) ions. To confirm the presence of plateau-generating mechanisms, we performed several key tests (Russell and Hartline 1982), including the trigger test, termination test, threshold test, all-or-none test, and symmetrical pulse test. We showed that brief pulses of depolarizing current trigger a plateau state and that plateau potentials can be terminated using brief pulses of hyperpolarizing current. Triggering and terminating of plateau potentials in these cells are threshold phenomena, and both triggering and terminating stimuli result in “all-or-none” responses that show no change in magnitude as stimulus intensity is increased.

**Rebound excitation can also trigger entry into a plateau state**

In cells in which plateau potentials could be triggered using the rebound excitation from prolonged pulses of hyperpolarizing current, time-dependent rectification produced a depolarizing sag toward the resting membrane potential during the hyperpolarizing pulse. Our results suggest that this rectification is caused by a slowly activating inward current that is activated by hyperpolarization. Slow deactivation of this current on cessation of the hyperpolarizing current pulse could underlie the rebound excitation that triggers the generation of a plateau potential, presumably through the activation of Ca\(^{2+}\) channels in the cells. Full characterization of this hyperpolarization-activated current in *Manduca* AL neurons awaits further investigation, but its properties, including its sensitivity to blockade by Cs\(^{+}\), resemble those of the hyperpolarization-activated inward current, \(I_h\), described already in many vertebrate and invertebrate neurons (Kiehn and Harris-Warrick 1992b; McCormick and Pape 1990; Pape 1996).

**Plateau potentials depend on the influx of Ca\(^{2+}\) ions**

Blocking Ca\(^{2+}\) currents in the cells with Cd\(^{2+}\) not only prevented the generation of plateau potentials but also abolished bursts of electrical activity in developing *Manduca* AL neurons exhibiting bursts of electrical activity also responded to 5HT with increases both in the number of spikes and in the number and duration of plateau potentials recorded in the cells (Fig. 8B).

**TABLE 3. Plateau potentials resulting from Ca\(^{2+}\) currents in isolation**

<table>
<thead>
<tr>
<th>RMP, mV</th>
<th>Plateau Potential, mV</th>
<th>Plateau Amplitude, mV</th>
<th>Plateau Duration, ms</th>
<th>†Rate of Repolarization, mV/ms</th>
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<tbody>
<tr>
<td>−50 ± 4.5 (4)</td>
<td>23 ± 16 (4)</td>
<td>73 ± 18 (4)</td>
<td>567 ± 194 (4)</td>
<td>0.28 ± 0.03 (4)</td>
</tr>
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</table>

Values are mean ± SD. *Resting membrane potential (RMP) retained at control levels using current injection. †Measured from termination of plateau to 50% repolarization to resting level.

**FIG. 8. Promotion of plateau potential formation by 5HT. A:** recording from a cell late in metamorphosis (stage 16) maintained 6 days in vitro. Brief pulses of depolarizing current alone fail to trigger plateau potentials in the neuron (Ai). Bath application of 5HT (50 μM) promoted the cell’s entry into a stable depolarized (plateau) state. Bi: electrical activity recorded from a stage-12 AL neuron in situ in a semi-intact brain preparation. Recordings were obtained in 3-s episodes using pClamp6 acquisition software with a minimum (0) time interval selected between episodes. Ten successive episodes have been superimposed in each panel to show the spontaneous appearance of electrical activity over time. Activity is characterized by prolonged periods of membrane depolarization (plateau potentials) carrying bursts of action potentials. Bii: increased cell excitability following exposure of the preparation to 5HT (50 μM). 5HT was bath applied for 2.5 min prior to taking this 30-s recording. The preparation was immediately washed in 5HT-free saline. Biii: spontaneous activity recorded after washing the preparation for 10 min in 5HT-free saline.
neurons. This indicates that both depend on the activation of Ca\(^{2+}\) channels in the cells. Our results indicate, however, that, in contrast to the formation and maintenance of plateau potentials, termination of the plateau state is not Ca\(^{2+}\)-mediated. Replacement of Ca\(^{2+}\) with Ba\(^{2+}\) reduced the duration of plateau potentials, suggesting that their termination is unlikely to involve Ca\(^{2+}\)-mediated inactivation of Ca\(^{2+}\) channels (e.g., Gutnick et al. 1989). Our results suggest also that termination of plateau potentials can occur independently of K\(^+\) channel activation, because blocking K\(^+\) currents with TEA reduced the duration of the plateau state and increased rather than decreased the rate of membrane repolarization. It seems likely that, in the absence (or severe reduction) of K\(^+\) channel activation, sustained depolarization inactivates Ca\(^{2+}\) currents that maintain the plateau state.

While activation of K\(^+\) channels does not seem to be essential for terminating plateau potentials, we assume that, under normal conditions, K\(^+\) channel activation will contribute to membrane repolarization. In the presence of TEA, however, the voltage at which the membrane commences rapid repolarization (the plateau inactivation voltage or “breakpoint”) is determined primarily by the voltage dependence of the Ca\(^{2+}\) channels (Reuveni et al. 1993). Ca\(^{2+}\) currents in Manduca AL neurons activate around −40 mV and peak around −10 mV (Mercer and Hildebrand 2002b; Mercer et al. 1995). They are characterized by relatively slow inactivation but undergo steady-state inactivation, being 100% available at −100 mV, 50% available at approximately −40 mV, and fully inactivated at −10 mV and above. The voltage-dependent activation and inactivation properties of these currents are similar to those of Ca\(^{2+}\) currents described in other insect preparations, including fruit fly Drosophila melanogaster (Baines and Bate 1998; Byerly and Leung 1988), cricket Gryllus bimaculatus (Kloppenburg and Horner 1998), and honey bee Apis mellifera (Grünewald 2003; Kloppenburg et al. 1999; Schäfer et al. 1994). Plateau inactivation voltages recorded in this study under TEA (−7.25 ± 4.6 mV) and for Ca\(^{2+}\) currents in isolation (−5.53 ± 0.4 mV) are consistent also with the voltage-dependent properties reported for Ca\(^{2+}\) currents in developing Manduca leg motoneurons (Grünewald and Levine 1998; Hayashi and Levine 1992), as well as in AL neurons of the moth (Mercer and Hildebrand 2002b; Mercer et al. 1995). Taken together, our results indicate that voltage-activated Ca\(^{2+}\) currents contribute fundamentally to the bistable properties of developing Manduca AL neurons.

Persistent or slowly inactivating Ca\(^{2+}\) currents contribute in a similar way to the maintenance of depolarized plateau potentials in vertebrate neurons (e.g., Carlin et al. 2000; Perrier and Hounsgaard 2000; Seamans et al. 1997; Vergara et al. 2003) and the bistable properties of these neurons allow transient depolarizing inputs, including synaptic inputs, to produce prolonged depolarizations and sustained periods of spiking activity (Kiehn and Eken 1998; Reuveni et al. 1993). Slowly inactivating Na\(^{+}\) currents can play a similar role (Hsiao et al. 1998; Larkum et al. 2001; Schwindt and Crill 1998). However, while there is preliminary evidence to suggest that persistent Na\(^{+}\) currents are expressed in some insect neurons (e.g., Mercer and Hildebrand 2002b; Schäfer et al. 1994), we could find no evidence in this study that Na\(^{+}\) currents play a role in the formation or maintenance of plateau potentials in Manduca AL neurons. Neither the amplitude nor the duration of plateau potentials was altered by removal of Na\(^{+}\) ions from the external medium or by blocking Na\(^{+}\) channels in these cells with TTX.

**5HT promotes the formation and maintenance of plateau potentials**

A readily identifiable 5HT-immunoreactive neuron invades developing AL glomeruli at pupal stage 6 (Kent et al. 1987; Oland et al. 1995). Ultrastructural studies have shown that, within the glomeruli of adult ALs, most contacts involving this neuron are output synapses (Sun et al. 1993). 5HT applied exogenously to AL neurons in vitro, in situ in semi-intact brain preparations, and in vivo in the brain of the adult moth increases the excitability of AL neurons, exerting its effects through the modulation of K\(^{+}\) currents in the cells (Kloppenburg and Heinbockel 2000; Kloppenburg and Hildebrand 1995; Kloppenburg et al. 1999; Mercer et al. 1995, 1996b). Here we show that, while plateau potentials cannot be triggered by 5HT alone, this neuromodulator increases the likelihood that depolarizing current pulses (and presumably synaptic input) will trigger a cell's entry into a plateau state. This is reminiscent of other cell types, including vertebrate motoneurons that, in the presence of neuromodulators such as 5HT and norepinephrine, can be shifted between two stable modes of firing (Eken and Kiehn 1989; Hounsgaard and Kiehn 1989). This property endows the neurons with a mechanism for translating brief synaptic inputs into long-lasting motor output (reviewed by Kiehn and Eken 1998). 5HT has been shown to increase the excitability of motoneurons in several ways, including through the enhancement of the inward rectifier current, \(I_{\text{K}}\) (Kjaerulf and Kiehn 2001). Plateau properties in crustacean motor neurons can also be induced through a dual-conductance mechanism involving 5HT modulation of hyperpolarization-activated inward current, \(I_{\text{f}}\), and Ca\(^{2+}\)-dependent outward current (Kiehn and Harris-Warrick 1992a,b). We have shown elsewhere that, in Manduca AL neurons, 5HT modulates a transient A-type current as well as a sustained K\(^{+}\) current that resembles the delayed rectifier, \(I_{\text{KV}}\) (Kloppenburg et al. 1999; Mercer et al. 1995, 1996b). However, effects of 5HT on Ca\(^{2+}\)-activated K\(^{+}\) currents and the hyperpolarization-activated inward current observed in this study have yet to be determined. Whether 5HT modulates Ca\(^{2+}\) currents in developing Manduca AL neurons that show bursts of electrical activity also remains unclear.

**Functional significance**

There is compelling evidence that early forms of electrical excitability regulate neuronal growth and differentiation (e.g., Baines et al. 2001; Gu and Spitzer 1980; Kater and Mills 1991; Kater et al. 1988; Schilling et al. 1991; Spitzer et al. 1995, 2002) and contribute also to activity-dependent tuning of neuronal connections (e.g., Katz and Shatz 1996; Ruthazer and Stryker 1996; Shatz 1994; Sherrard and Bower 1998). In embryonic Periplaneta neurons, as in vertebrate neurons (e.g., Gallo et al. 1987; Koike et al. 1989; Toescu 1999), Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels influences both the survival and differentiation of neurons in culture (Benquet et al. 2001), and in Manduca, elegant studies by Duch and Levine (2000, 2002) suggest that Ca\(^{2+}\) spikes play a key role in
postembryonic dendritic remodeling of motor neurons. Here we show that developing AL neurons generate Ca\(^{2+}\)-mediated plateau potentials and that influx of Ca\(^{2+}\) ions underlies the characteristic bursts of electrical activity in these cells. The bistable properties of Manduca AL neurons should enable transient depolarizing synaptic inputs to produce prolonged depolarizations and sustained periods of spiking activity, which may also occur spontaneously without exogenous trigger.

Changes in intracellular Ca\(^{2+}\) levels resulting from such activity could trigger a diverse array of cellular responses, ranging from modulation of ion channels (Gutnick et al. 1989) to regulation of neuronal gene expression (Berriedge 1998; Bito et al. 1997; Benneschitz and Katz 2001; Finkbeiner and Greenberg 1998). It seems likely that sustained electrical activity in ALs of the moth during critical periods of glomerulus formation and synaptogenesis contribute to the development of this highly structured neuropil. Our results add support also to the growing body of evidence that serotonin contributes to both the development and plasticity of AL neurons in the moth.

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