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GABA-mediated synaptic inhibition of projection neurons in the antennal lobes of the sphinx moth, *Manduca sexta*

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Summary. 1. Responses of neurons in the antennal lobe (AL) of the moth *Manduca sexta* to stimulation of the ipsilateral antenna by odors consist of excitatory and inhibitory synaptic potentials (Fig. 2A). Stimulation of primary afferent fibers by electrical shock of the antennal nerve causes a characteristic IPSP-EPSP synaptic response in AL projection neurons (Fig. 2B).

2. The IPSP in projection neurons reverses below the resting potential (Fig. 3), is sensitive to changes in external (Fig. 4) and internal (Fig. 5) chloride concentration, and thus is apparently mediated by an increase in chloride conductance.

3. The IPSP is reversibly blocked by 100 μM picrotoxin (Fig. 6) or bicuculline (Fig. 7).

4. Many AL neurons respond to application of GABA with a strong hyperpolarization and an inhibition of spontaneous spiking activity (Fig. 8). GABA responses are associated with an increase in neuronal input conductance (Fig. 9) and a reversal potential below the resting potential (Fig. 11).

5. Application of GABA blocks inhibitory synaptic inputs (Fig. 12A) and reduces or blocks excitatory inputs (Fig. 12B). EPSPs can be protected from depression by application of GABA (Fig. 12B).

6. Muscimol, a GABA analog that mimics GABA responses at $GABA_A$ receptors but not at $GABA_B$ receptors in the vertebrate CNS, inhibits many AL neurons in the moth (Fig. 13).

Introduction

Gamma-aminobutyric acid (GABA) functions as an inhibitory neurotransmitter in both vertebrate and invertebrate nervous systems. There are pharmacological dissimilarities, however, between vertebrate and invertebrate neuronal GABA receptors (Simmonds 1983). At present there is insufficient information about the functions and pharmacology of insect GABA receptors to make very detailed comparisons with vertebrate GABA receptors. In this study, we examine GABA-mediated synaptic inhibition of neurons in the olfactory pathway of the moth *Manduca sexta* as one model in which to study the pharmacology of GABA receptors in the CNS of insects.

The primary olfactory centers in the brain of adult *Manduca* are the antennal lobes (ALs) of the deutocerebrum (see Hildebrand 1985). Each AL receives axonal projections, via the antennal nerve, from sensory neurons in the ipsilateral antenna. The AL contains approximately 60 glomeruli, condensed areas of neuropil in which axons from sensory neurons terminate and all synaptic contacts in the ALs have been found (Tolbert and Hildebrand 1981). Two principal morphological classes of AL neurons have been described in *Manduca*: local interneurons, which send processes into most or all of the glomeruli in the AL but are restricted entirely to the AL, and projection neurons, which send an axon into the protocerebrum (Matsumoto and Hildebrand 1981).

We have begun to analyze the physiology of olfactory information processing in the brain of *Manduca*. Many local and projection neurons have been characterized with respect to their responses to stimulation of antennal receptors with behaviorally relevant odors (Matsumoto and Hildebrand 1981; Christensen and Hildebrand 1987b). Much of the synaptic activity recorded in AL neurons is inhibitory (Matsumoto and Hildebrand 1981; Harrow and Hildebrand 1982), and several lines of evidence indicate that GABA is probably used as a neurotransmitter by many AL neurons. Neurochemical analysis has revealed that GABA is

Abbreviations: *AL* antennal lobe; *BMI* bicuculline methiodide; *IACT* inner antenno-cerebral tract; *LY* Lucifer Yellow

synthesized from glutamate and stored in the ALs (Maxwell et al. 1978; Kingan and Hildebrand 1985). More recently, polyclonal antibodies raised against GABA have been shown to label all of the AL glomeruli, as well as many somata in the ALs (Hoskins et al. 1986). Using a double fluorescence labelling procedure, GABA immunoreactivity was demonstrated primarily in cells individually identified as local interneurons (Hoskins et al. 1986).

Recently two classes of GABA receptors have been recognized in vertebrate CNS based on differences in pharmacological specificity (Bowery et al. 1983; Simmonds 1983). One class of GABA receptors, designated $GABA_A$, mediates inhibition via a chloride conductance that is antagonized by picrotoxin and bicuculline and is activated by GABA and the GABA analog muscimol (DeFeudis 1977; Enna 1983). The other class, $GABA_B$ receptors, also mediates inhibition but may be functionally coupled to potassium or calcium channels (Dunlap 1981; Newberry and Nicoll 1984). $GABA_B$ receptors are insensitive to bicuculline, and the GABA analog baclofen is a potent agonist (Bowery et al. 1983; Newberry and Nicoll 1984). The physiological properties of insect CNS GABA receptors generally resemble those of vertebrate $GABA_A$ receptors, in that inhibition is mediated by chloride (Kerkut et al. 1969; Pitman and Kerkut 1970; Hue and Callec 1983), picrotoxin and bicuculline are antagonists (Pitman and Kerkut 1970; Walker et al. 1971; Callec 1974; Hue and Callec 1983; Gregory et al. 1985; Wafford and Sattelle 1986) and muscimol is a potent agonist (Roberts et al. 1981; Gregory et al. 1985). There is as yet, however, insufficient information to ascertain whether GABA receptors in insects (or other invertebrates) form a heterogeneous population, or to understand fully how insect GABA receptors may be different from those of vertebrates.

In this study we probed inhibitory synaptic interactions among AL neurons of morphologically distinct classes to explore further the characteristics of insect CNS GABA receptors. This preparation provides three important advantages for studies of inhibitory synaptic potentials in the insect CNS: 1) observations of PSPs and responses to applied GABA are made from neuropil processes rather than a remote and presumedly non-synaptic cell soma, 2) the IPSP studied occurs in a morphologically and physiologically distinct class of CNS neurons, and 3) the IPSP is a reproducible, physiological response. We have found that this synaptic inhibition in the ALs of *Manduca sexta* is chloride-dependent, is blocked by bicuculline and picrotoxin, and can be mimicked by application of GABA or muscimol. Our results indicate that the GABA receptors mediating this inhibition are pharmacologically more similar to the $GABA_A$ receptors than to $GABA_B$ receptors in vertebrate CNS. A preliminary account of some parts of this study was reported previously (Christensen et al. 1985).

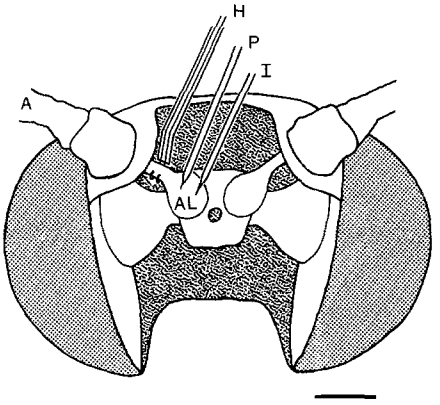

Fig. 1. Schematic drawing of the preparation, showing frontal view of the head with cuticle and muscles removed. *AL* antennal lobe; *A* antennal flagellum; *H* hook electrodes under antennal nerve; *I* intracellular electrode; *P* pressure ejection electrode. Scale marker 500 μm

Materials and methods

Manduca sexta were reared from eggs in the laboratory on an artificial diet (Bell and Joachim 1976) under a 17:7 L:D cycle, as described by Sanes and Hildebrand (1976). Male and female adults, 1–4 days post-eclosion, were used in these studies. After an animal had been chilled on ice and immobilized in a plastic tube, the brain was exposed by removing the palps, proboscis, cibarial pump, and antennal muscles. The head was then removed and pinned onto a wax-coated dish. A flattened hypodermic needle served as a platform to stabilize the brain and also as a conduit for superfusion of saline solutions. A pair of silver wire hook electrodes, placed under the antennal nerve near its entrance to the brain, was used to stimulate the nerve electrically (Fig. 1). The AL to be studied was desheathed surgically to provide easier access to the neuropil both for penetration with intracellular electrodes and for superfusion of pharmacological agents.

Normal saline consisted of 149.9 m*M* NaCl, 3.0 m*M* KCl, 3.0 m*M* $CaCl_2$, 10.0 m*M* N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer (pH 6.9), and 25.0 m*M* sucrose for osmotic balance with the extracellular fluid (modified from Pichon et al. 1972). A superfusion system with a small closed reservoir provided a constant flow of the superfusate even when input reservoirs were switched during pharmacological manipulations. Low-Cl^- saline had some SO_4^{-2} substituted for Cl^-, and consisted of 112.4 m*M* NaCl, 18.7 m*M* Na_2SO_4, 3.0 m*M* KCl, 3.0 m*M* $CaCl_2$, 10.0 m*M* TES buffer (pH 6.9), and 43.7 m*M* sucrose. Thus the Cl^- concentration was 121.4 m*M*, or 24% lower than normal saline (158.9 m*M* Cl^-). Solutions of picrotoxin, picrotoxinin and bicuculline methiodide (BMI) (all from Sigma) were made by adding the required agent to normal saline to a final concentration of 100 μ*M*. Sa-

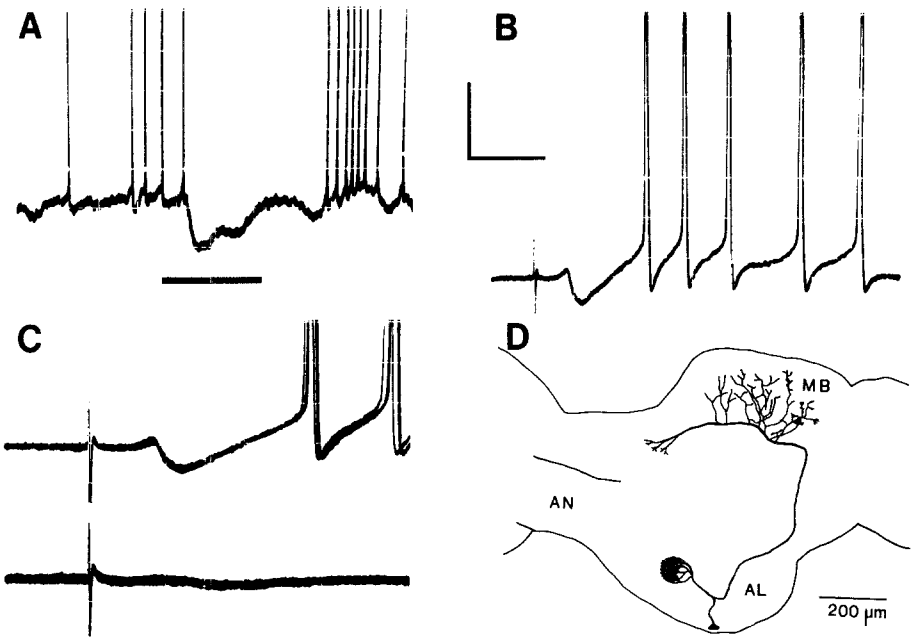

Fig. 2A–D. Synaptic inhibition in projection neurons. **A** Inhibitory response to presentation of *E*-2-hexen-1-ol, a common leaf alcohol, to the ipsilateral antenna; stimulus marker under trace. Peaks of the spikes in this and all subsequent records are clipped. **B** Response of a projection neuron to electrical shock of the antennal nerve; stimulus at artifact. **C** Responses to multiple antennal nerve shocks (overlayed oscilloscope traces). Top: 1 Hz stimulation, three successive stimuli. Bottom: 10 Hz stimulation, third, fourth and fifth stimuli. **D** Camera lucida drawing of LY-stained projection neuron viewed in whole mount, dorsal view. This cell has processes in a single ordinary glomerulus and an axon in the inner antenno-cerebral tract, and thus is classified as a P1(U2:G) cell according to Christensen and Hildebrand (1987a). *AN* antennal nerve; *AL* antennal lobe; *MB* calyces of the mushroom bodies. **A**, **B–C**, and **D** are from three different preparations. Scale: **A** 10 mV, 400 ms; **B** 20 mV, 20 ms; **C** 20 mV, 10 ms

line solutions of GABA or muscimol were pressure-ejected from a large-tipped (20 μm diameter) glass micropipette inserted into the AL neuropil. Ejection pressure (13 p.s.i) and duration were controlled by a Picospritzer (General Valve Corp.). The GABA solution consisted of 100 m*M* GABA in normal saline without sucrose (the GABA already overcompensated for osmotic balance). Muscimol (Sigma) was added to normal saline (without sucrose) to a concentration of 30 m*M*.

A continuous stream of charcoal-filtered air was directed over the ipsilateral antenna. To stimulate the antenna with odors, a solenoid-driven valve was activated, which diverted the airstream from a clean glass tube to a glass cartridge containing a piece of filter paper impregnated with an odorant (adapted from Waldow 1977). An electrical pulse (0.1 ms, 5–50 V) applied to the hook electrodes under the antennal nerve often substituted for olfactory stimulation of primary afferent inputs (Harrow and Hildebrand 1982; Christensen and Hildebrand 1987b).

Intracellular electrodes were filled with 2.5 *M* KCl or 2.5 *M* potassium acetate (KAc) and had resistances of 40–150 MΩ. Lucifer Yellow (LY; Aldrich; 4% aqueous solution) was used to stain impaled neurons. The resistances of LY-containing electrodes were 175–400 MΩ. LY was injected into cells with 10 nA-min of iontophoretic current. The brains were fixed with 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4), dehydrated through an ethanol series, cleared with methyl salicylate, and viewed in whole-mount with a fluorescence microscope.

Standard intracellular recording and stimulation techniques were used. An active bridge electrometer was used to record voltage and inject current simultaneously. Current pulses (0.5–1 s) were used to measure reversal potentials of PSPs evoked by nerve shock and GABA application. Absolute resting membrane potentials could generally not be measured accurately in the AL neuropil owing to the dense packing of the neuropil, which prevented the true transmembrane potential from being measured as an electrode entered a cell, and to time-limited impalements of fine processes so that electrodes ordinarily were not withdrawn from cells purposely to measure resting potentials. Therefore reversal potentials were measured, and are reported here, with respect to the resting potential.

Results

All neurons were impaled in the AL neuropil, apparently in or near regions of active membrane, as action potentials were usually 60–80 mV in amplitude. Large (5–15 mV) IPSPs and EPSPs were often recorded, and their amplitudes could be affected by small amounts (<1 nA) of injected current. All AL neurons responded to olfactory stimulation of the ipsilateral antenna, although some showed selectivity in their response spectra (data not shown). Many AL neurons were inhibited, with or without accompanying excitation, in response to the presentation of behaviorally relevant odors (Fig. 2A). All AL neurons also responded to delivery of a brief electrical shock (0.1 ms, 10–50 V) to the ipsilateral antennal nerve. We

found that the response to electrical shock of the antennal nerve was a good predictor of the responses of the cell to stimulation of the antenna with odors, and thus the response to nerve shock was used as a physiological descriptor of neurons.

One commonly observed pattern of response to electrical shock of the antennal nerve consisted of a small depolarizing 'bump', followed by an IPSP and then an EPSP which generally gave rise to spikes (Fig. 2B) (Harrow and Hildebrand 1982). Both inhibitory and excitatory response phases showed rapid decrement during repetitive stimulation at frequencies above 5 Hz (Fig. 2C), suggestive of a polysynaptic pathway from primary afferents to these neurons. LY staining of AL neurons with this response profile revealed projection neurons with uniglomerular dendritic arborizations and axons which project via the inner antennocerebral tract (IACT; Hoskins et al. 1986) to the calyces of the mushroom bodies and further to the lateral protocerebrum (Fig. 2D). Two other morphological types of AL projection neurons, which project to the protocerebrum via the middle and outer antenno-cerebral tracts, have been described (Hildebrand and Montague 1986; Hoskins et al. 1986; U. Homberg, R. Montague, J. Hildebrand, in preparation), but we have not yet impaled and recorded from these cells, and their physiological properties are still unknown. Therefore we use the term 'projection neuron' in this paper to refer exclusively to IACT-type projections neurons (Fig. 2D).

In 33 neurons in which the IPSP-EPSP response profile was observed, intracellular staining always revealed a projection neuron similar to the one shown in Fig. 2D. With the exception of some projection neurons that have arborizations in the male-specific macroglomerular complex (the site of central processing of primary afferent information about female sex pheromones; Matsumoto and Hildebrand 1981; Christensen and Hildebrand 1987a, b), none of the projection neurons we have stained with dye have shown any other pattern of responses to antennal nerve shock. Furthermore, none of the morphologically characterized local interneurons exhibited this type of response pattern upon stimulation of the antennal nerve. Thus we used the IPSP-EPSP response (Fig. 2B) as a physiological marker for identifying projection neurons. Most experiments were performed with KCl or KAc electrodes, since the use of LY-filled electrodes was not necessary to identify projection neurons.

The IPSP in projection neurons evoked by electrical stimulation of the antennal nerve was easily

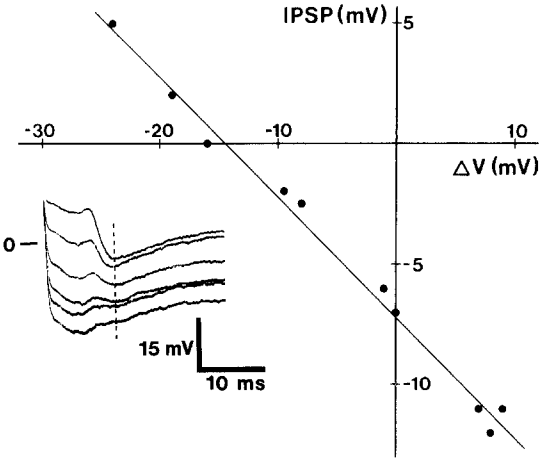

Fig. 3. Reversal potential of IPSP in a projection neuron, produced by electrical stimulation of the antennal nerve. Graph of IPSP peak amplitude vs membrane potential (relative to rest). IPSP amplitude was measured from the voltage just prior to the 'bump' to the peak of the IPSP. Line is least-squares regression, interpolated reversal potential = −14.5 mV. Inset: Current injection and nerve shock. Resting potential indicated by 0, dotted line shows time of peak IPSP amplitude. Nerve shock occurs at start of traces

reversed by injection of hyperpolarizing current into the neurons. Figure 3 shows the results of one experiment in which the reversal potential of the IPSP was −14.5 mV with respect to the resting potential. Note that while the EPSP was also affected by injected current, the depolarizing 'bump' was unaffected (Fig. 3, inset), indicating that it may not result from chemical synaptic input but perhaps is an ephaptic representation of synchronous firing of nearby neurons. Reversal of an IPSP below the resting potential implies the involvement of either Cl^- or K^+ ions, or both. Because there is considerable evidence that GABA is a neurotransmitter in the ALs (see Introduction), we investigated the pharmacology of the IPSP in projection neurons, testing agents known to affect GABA-mediated synaptic transmission.

We examined the involvement of chloride ions in the production of the IPSP by changing the Cl^- equilibrium potential. We impaled projection neurons while superfusing the preparation with normal saline, measured the IPSP following antennal nerve shock, and then switched the superfusate to low-Cl^- saline. This caused the IPSP to reverse and become excitatory (Fig. 4) ($n=4$). Returning to normal saline restored the IPSP to its original hyperpolarizing state. We next injected Cl^- ions into projection neurons using KCl-filled electrodes. In most projection neurons the IPSP was not affected by injection of Cl^- ions, but in three neurons the IPSP was reversed following Cl^- injec-

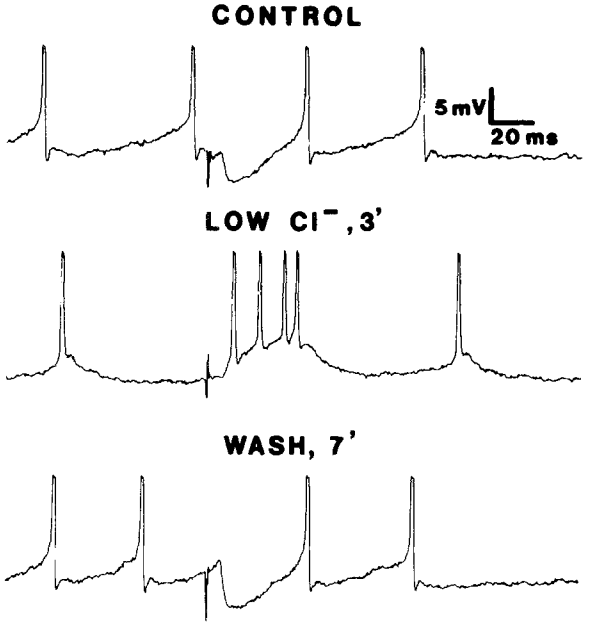

Fig. 4. Low-Cl^- saline reverses IPSP. Top: Control IPSP in normal saline. Middle: IPSP is reversed after 3 min of superfusion with low-Cl^- saline. Bottom: IPSP has returned to control state after 7 min wash with normal saline

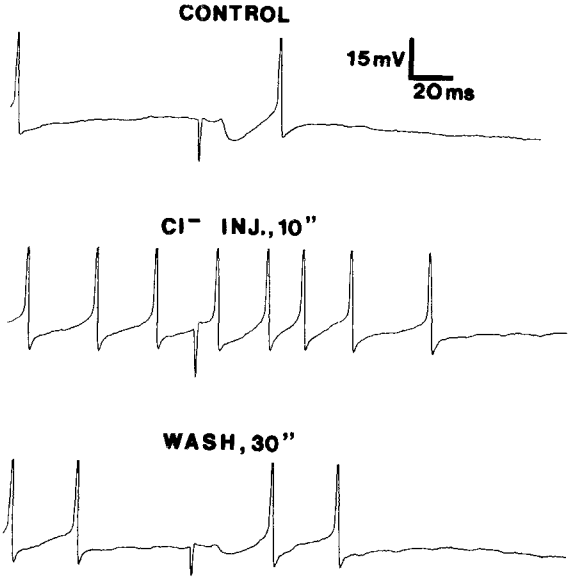

Fig. 5. Cl^- injection shifts IPSP reversal potential. Top: Control IPSP before Cl^- injection. Middle: IPSP reversal potential has shifted to ca. resting potential 10 s after releasing 10 min of -1 nA injected current (Cl^- injection). Bottom: IPSP reversal potential has returned to near control value 30 s after releasing injection current

tion, and it returned to a hyperpolarizing potential within 30 s after cessation of the Cl^- injection current (Fig. 5). Because both low-Cl^- saline and (in a few neurons) injection of Cl^- changed the IPSP into an EPSP, it appears that the IPSP is caused by an increased Cl^- conductance.

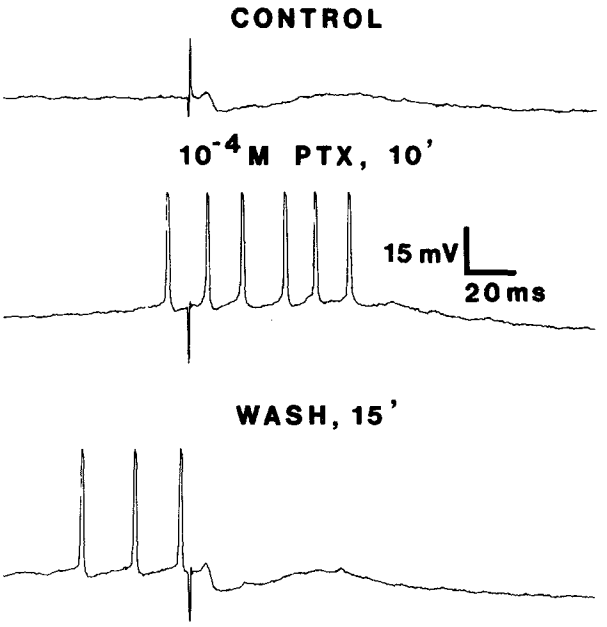

Fig. 6. Picrotoxin blocks projection neuron IPSP. Top: Control IPSP in normal saline. Middle: IPSP is blocked after 5 min of superfusion with saline containing 100 μM picrotoxin, revealing underlying excitation. Bottom: Recovery of IPSP 15 min after changing to normal saline

Picrotoxin is known to block Cl^--mediated IPSPs in many vertebrate and invertebrate neurons (Constanti 1978). In projection neurons, 100 μM saline solutions of picrotoxin or picrotoxinin (the active component of picrotoxin) were effective in blocking the IPSP (Fig. 6) ($n=6$). The EPSP following the IPSP was not blocked by picrotoxin and the IPSP was replaced by excitation (Fig. 6). The effect was reversible by washing in normal saline. Both picrotoxin and picrotoxinin took up to 20 min to block the IPSP and longer to be washed out, and they often caused some side effects such as an increased tendency of neurons to exhibit bursting activity.

GABA-induced responses in invertebrates, and those mediated by $GABA_A$ receptors in vertebrates, are antagonized by the alkaloid bicuculline (Nistri and Constanti 1979). We used a water-soluble form of this agent, bicuculline methiodide (BMI) (Olsen et al. 1976), to test for bicuculline antagonism in our preparation. Superfusion with saline containing 100 μM BMI blocked the IPSP in projection neurons quickly and reliably, and the effect was easily reversed by washing in normal saline (Fig. 7) ($n=15$). A reduction in IPSP amplitude generally began after about 1.5 min in BMI saline solution, and the IPSP was completely blocked after about 3 min. In all cases, excitation with spiking replaced the IPSP and the following EPSP was not affected (Fig. 7). Restoration of the

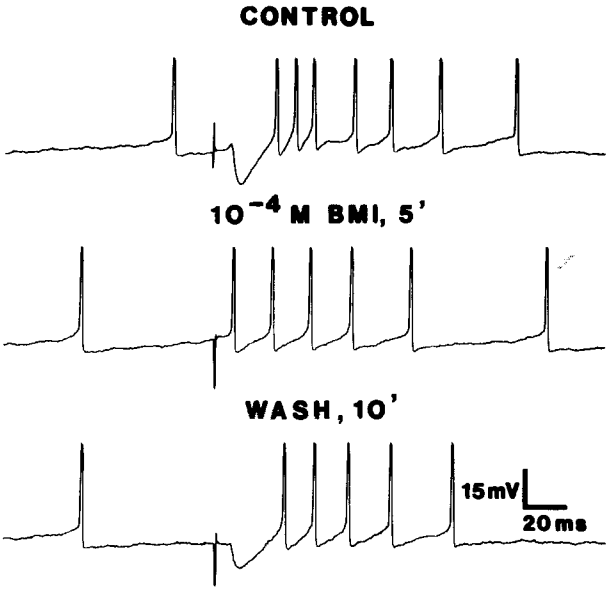

Fig. 7. Bicuculline methiodide blocks projection neuron IPSP. Top: Control IPSP in normal saline. Middle: IPSP is blocked after 5 min of superfusion with saline containing 100 μ*M* BMI, leaving underlying excitation. Bottom: Recovery of IPSP 10 min after returning to normal saline

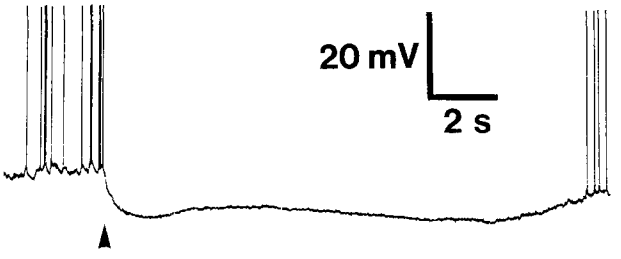

Fig. 8. Response of interneuron to GABA. A 20 ms, 13 psi pressure pulse applied to the pressure ejection electrode containing saline with 100 m*M* GABA. Arrow shows time of stimulus

IPSP required washing with normal saline for about 5 min. BMI was effective in reversibly blocking the IPSP in all projection neurons on which it was tested.

We studied the effects on neurons of injecting GABA into the neuropil of the AL to verify that exogenously applied GABA can elicit inhibitory responses in AL neurons and to compare such responses with neurally evoked IPSPs. GABA introduced into the neuropil of the AL by pressure ejection caused hyperpolarization and inhibition of background spiking in 26 AL neurons (out of about 50 tested) (Fig. 8). The latencies from the beginning of the pressure pulse (10–100 ms duration) to the onset of hyperpolarization were in the range of 50–100 ms. All GABA-sensitive neurons showed an apparently monophasic GABA response. The hyperpolarization was accompanied

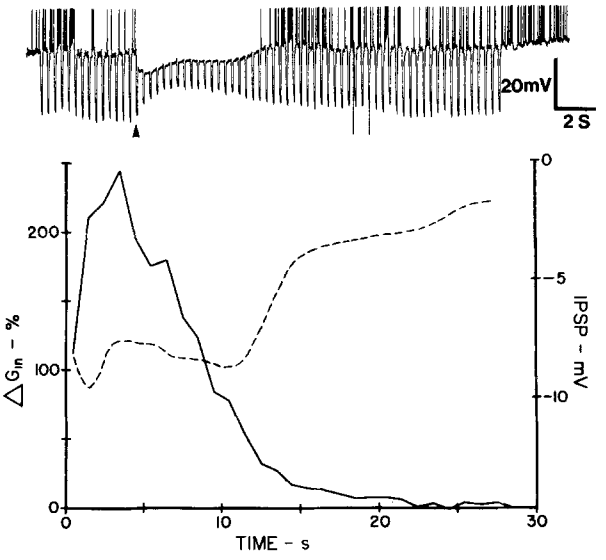

Fig. 9. GABA increases neuronal input conductance. Top: Constant current pulses injected into the neuron demonstrate input conductance increase following GABA pressure ejection stimulus (at arrow). Bottom: Solid line is change in input conductance (average of 5 trials), relative to pre-stimulus control input conductance, as a function of time following GABA pulse. Dashed line shows membrane voltage during GABA response (single trial)

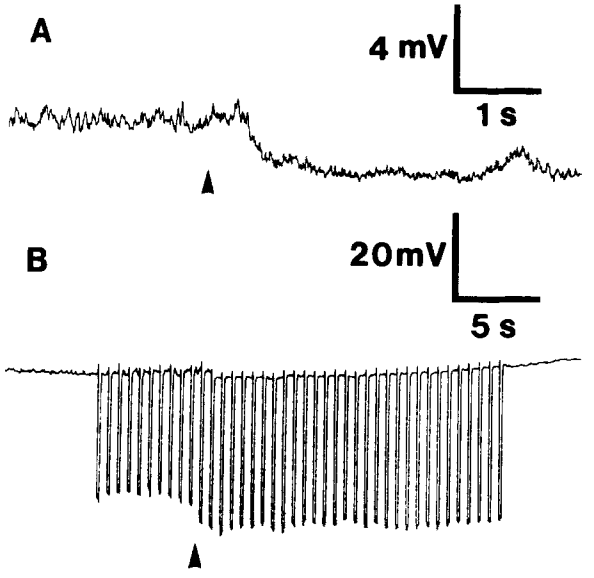

Fig. 10A, B. GABA application can cause input conductance decrease. **A** Response to GABA application (stimulus at arrow) showing small hyperpolarization. **B** Injection of constant current pulses demonstrates a decrease in input conductance following GABA application

by an increase in input conductance, in the range of 20–200% of the resting value (Fig. 9), except in one neuron in which the GABA-induced hyperpolarization was associated with a small (15%) but consistent decrease in input conductance (Fig. 10).

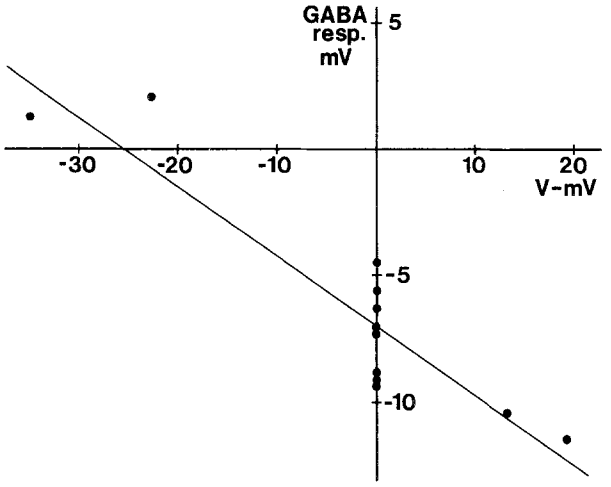

Fig. 11. GABA reversal potential. Graph of peak voltage response to GABA application during current injection into interneuron vs. change in membrane potential due to current. Line is least-squares regression. Interpolated reversal potential, −25.2 mV, is at end of range of values seen in all cells tested

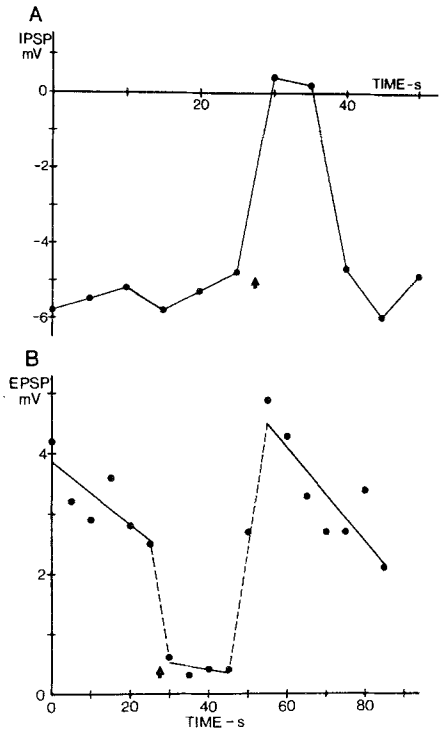

Fig. 12A, B. GABA blocks responses to antennal nerve shock. **A** Graph of effect of GABA application on antennal nerve shock responses. Points are IPSP amplitudes for nerve shocks delivered at 0.2 Hz. GABA application at arrow. **B** GABA application protects antennal nerve shock-induced EPSP from depression. Antennal nerve stimulation at 0.2 Hz produces EPSPs which are reduced greatly during GABA application. As GABA response ends, EPSP temporarily recovers to a greater amplitude than before GABA application. Solid lines are least-squares regressions for data before, during and after GABA application

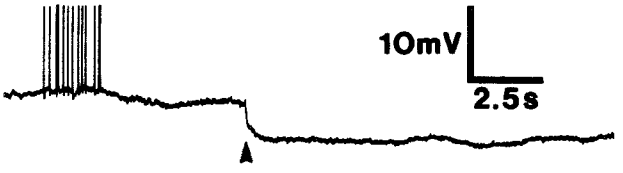

Fig. 13. Muscimol inhibits AL neurons. Response of a neuron to 30 m*M* muscimol in pressure ejection electrode (stimulus at arrow). Strong hyperpolarization and inhibition of spontaneous spiking followed muscimol application

The large conductance increases generally observed during responses to GABA made it difficult to induce reversals of those responses with injected current. In several AL neurons in which the GABA response could be reversed, the reversal potentials were 10–25 mV below the resting potential (Fig. 11) ($n=3$), and thus resembled the reversal potentials demonstrated for IPSPs evoked by electrical stimulation of the antennal nerve (Fig. 3). Note that the reversal potential shown in Fig. 11 was the largest value observed for a GABA response.

Application of GABA generally reduced or eliminated the responses (inhibitory and excitatory) of AL neurons to stimulation of the antennal nerve with electrical shock (Fig. 12). In a few neurons that exhibited EPSPs following electrical stimulation of the antennal nerve and which showed response decrement during stimulation at 0.2 Hz, the responses were apparently protected from depression by application of GABA. That is, after recovery from GABA-induced block, the EPSP amplitude temporarily increased to a greater level than that observed immediately before the application of GABA (Fig. 12B). In the absence of GABA, the amplitude of the EPSP would have continued to decline during 0.2 Hz stimulation and it would have been smaller at the time marked 60 s in Fig. 12B, for example.

Inhibition of AL neurons by GABA does not by itself indicate the nature of the receptors mediating the inhibition (e.g., similar to either $GABA_A$ or $GABA_B$). We examined the pharmacology of the receptors mediating the responses to applied GABA by using muscimol, an analog of GABA that is a potent agonist at vertebrate $GABA_A$ receptors. Muscimol is only weakly effective at $GABA_B$ receptors (Newberry and Nicoll 1984). In three preparations, muscimol strongly inhibited 7 of 8 neurons tested (Fig. 13). The hyperpolarizations observed were more persistent than those obtained with GABA, even though the concentration of muscimol in the pressure ejection

electrode (30 m*M*) was less than one-third the concentration of GABA (100 m*M*) in electrodes used for GABA application. This suggests that muscimol effectively activates receptors that mediate inhibition in AL neurons and that it may be more potent than GABA at doing so.

Discussion

A growing body of evidence indicates that GABA is a neurotransmitter in the ALs of *Manduca*. Biochemical studies have shown the presence and biosynthesis of GABA in the ALs (Maxwell et al. 1978; Kingan and Hildebrand 1985); anatomical techniques combined with immunocytochemical staining have revealed the cellular localization (primarily in local interneurons) of GABA in the ALs (Hoskins et al. 1986) and physiological evidence from intracellular recordings indicates that a great deal of inhibitory synaptic activity occurs in the ALs (Matsumoto and Hildebrand 1981; Harrow and Hildebrand 1982; Christensen and Hildebrand 1987b). We have begun to study these inhibitory synaptic potentials more closely using intracellular recordings combined with pharmacological manipulations.

IPSPs in AL projection neurons have pharmacological properties qualitatively similar to those of $GABA_A$ receptor-mediated IPSPs in the CNS of vertebrates. The IPSPs in AL projection neurons are associated with an increased conductance to Cl^- ions (Figs. 3–5), and are reversibly blocked by picrotoxin (Fig. 6) and bicuculline (Fig. 7). Application of GABA or muscimol inhibits AL neurons (Figs. 8, 13), increases their input conductance (Fig. 9), and reduces or eliminates synaptic inputs for the duration of the response (Fig. 12). While responses to applied GABA have not yet been examined using the same pharmacological manipulations described for the IPSP, we feel that these results provide strong evidence that GABA serves as an inhibitory synaptic transmitter in the ALs of *Manduca*, and in particular, mediates the IPSP observed in projection neurons following electrical shock of the antennal nerve.

In many insect CNS preparations, the use of KCl-filled intracellular electrodes often causes IPSPs to reverse even without passage of hyperpolarizing current (e.g. Kerkut et al. 1969; Pitman and Kerkut 1970). In this preparation, however, the IPSP in projection neurons was not reversed or reduced during impalement with KCl-filled electrodes. Furthermore, on only a few occasions did the IPSP reverse as a result of passing hyperpolarizing current using KCl electrodes (Fig. 5). This may be due to the structure of projection neurons and the site of impalement. The dendritic arbors are extensively branched and thus have a large volume. Impalements were made in the major process connecting the dendritic tree with the axon. Even though the electrodes were electrotonically close to the synapses, the large volumes presented by the nearby axon and the dendritic arbor may have hindered attempts to increase the intracellular Cl^- concentration by iontophoresis. Reversing the IPSP by changing extracellular Cl^- levels in the superfusate should not be affected by cell geometry, and indeed manipulations of external Cl^- concentration were always successful in affecting the IPSP.

In all but one case, hyperpolarizations induced by application of GABA were associated with an increase in input conductance (Fig. 9). The decreased conductance response to applied GABA shown in Fig. 10 could be due to a GABA receptor-mediated decrease in Na^+ conductance, but an explanation more consistent with the rest of our data is that GABA inhibited a neuron (or neurons) which provided steady-state excitatory input to the impaled neuron. This would cause a decrease in ongoing excitatory input which would appear as a hyperpolarization and decrease in steady-state input conductance.

Application of GABA reduces or blocks inhibitory synaptic responses in AL neurons (Fig. 12A). This finding is consistent with the idea that shock-induced IPSPs are mediated by GABA; during application of GABA, synaptic receptors would be occupied owing to the saturating concentration of GABA, and thus no further response could be elicited by synaptic release of GABA following nerve shock. However, shunting of synaptic current by a large GABA-induced postsynaptic conductance increase (Fig. 9) is also inherent in all of our measurements. Blocking of EPSPs by GABA (Fig. 12B) certainly is due in large part also to post-synaptic shunting. The increase in EPSP amplitude following recovery from block by GABA to a level greater than the pre-GABA amplitude (Fig. 12B) may indicate an additional presynaptic effect. If GABA is acting presynaptically to inhibit release of the excitatory transmitter, then the stimulus-dependent depression might be prevented, allowing recovery of the stimulus-release process and leading to a larger EPSP amplitude (see Bryan and Krasne 1977). Thus, while our findings do not directly address the issue of presynaptic inhibition, they do suggest that GABA mediates some presynaptic inhibition in the ALs.

The conclusions drawn from this and previous studies are beginning to provide us with information about the synaptic circuitry within the ALs. Our results indicate that the IPSP exhibited by projection neurons following electrical stimulation of the antennal nerve is mediated by GABA. We hypothesize that at least some GABA-containing local interneurons are synaptically excited by primary sensory fibers, and in turn the local interneurons synaptically inhibit projection neurons. The later EPSP in projection neurons may be due to ongoing excitatory input that is masked by the IPSP, or it could represent delayed excitatory input. Our data tend to support the former explanation, as both picrotoxin and bicuculline block the IPSP and reveal excitation in its place (Figs. 6 and 7), apparently unmasking excitatory input concomitant with the IPSP. The source of the excitation, whether primary afferent fibers or other interneurons, is not known; however, the fact that high frequency stimulation of the antennal nerve eliminates both the IPSP and EPSP in projection neurons (Fig. 2C) suggests that both phases of input are polysynaptic, mediated by interposed neurons.

The findings from this and other studies encourage a more complete characterization of GABA receptors in the insect CNS using many of the same criteria that have been established for vertebrate GABA receptors. One known difference between invertebrate and vertebrate $GABA_A$ receptors involves the nature of the antagonistic actions of bicuculline. At $GABA_A$ receptors in vertebrates, bicuculline is a competitive GABA antagonist (Simmonds 1982; 1983) that blocks GABA binding (Mann and Enna 1980; DeFeudis et al. 1980). In the insect CNS, bicuculline is generally a GABA antagonist (Fig. 7; Walker et al. 1971; Gregory et al. 1985), but in other invertebrates bicuculline antagonism has been shown to be noncompetitive (Takeuchi and Onodera 1972; Shank et al. 1974; Constanti 1978). Furthermore, bicuculline apparently does not block GABA binding to insect CNS tissue (Meiners et al. 1979; Mann and Enna 1980; Breer and Heilgenberg 1985; Lummis and Sattelle 1985; Lunt et al. 1985). Therefore the bicuculline and GABA binding sites on invertebrate GABA receptors appear to be independent in effect, but the two sites on vertebrate $GABA_A$ receptors are interdependent.

Another important property of $GABA_A$ receptors in vertebrates is their sensitivity to benzodiazepine drugs. GABA receptor-mediated responses as well as binding of [^{3}H]GABA are enhanced by benzodiazepines when $GABA_A$ receptors are involved, while no such effects are observed with $GABA_B$ receptors (Bowery et al. 1983). Benzodiazepine binding has been reported in insect CNS (Abalis et al. 1985; Lummis and Sattelle 1986; Robinson et al. 1986), and a preliminary report of enhancement of GABA-induced responses by benzodiazepines has appeared (Lees et al. 1985). Further experiments are needed to explore the possible effects of benzodiazepines, and the nature of bicuculline antagonism, in GABA-mediated synaptic inhibition in the ALs of *Manduca*.

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