Cholinergic Synaptic Transmission in Insect Mushroom Bodies In Vitro

SHARON OLESKEVICH
Research School of Biological Sciences, Australian National University, Canberra 2601, Australia

Oleskevich, Sharon. Cholinergic synaptic transmission in insect mushroom bodies in vitro. *J. Neurophysiol.* 82: 1091–1096, 1999. The mushroom body of the bee brain is an important site for learning and memory. Here we investigate synaptic transmission in the mushroom body using extracellular recording techniques in a whole bee brain in vitro preparation. The postsynaptic response showed attenuation by cadmium and paired-pulse facilitation, similar to in vivo findings. This confirms the viability of the in vitro preparation and supports the isolated whole bee brain as a useful model of the in vivo preparation. Bath application of the acetylcholine receptor antagonists, d-tubocurarine and α-bungarotoxin attenuated the postsynaptic response by 61% and 62% of control, respectively. The glutamate receptor antagonists, (+)-2-amino-5-phosphonopentanoic acid and 6-cyano-7-nitroquinoxaline-2,3-dione, had no effect. The invertebrate monoamine and neuromodulator, octopamine, transiently increased the postsynaptic response by 130% of control. These results suggest that synaptic transmission of the olfactory input pathway in the mushroom body is 1) mediated primarily by acetylcholine and 2) modulated by octopamine.

**INTRODUCTION**

The honeybee, *Apis mellifera*, has a highly developed olfactory learning capacity and relatively simple CNS and therefore is well suited for electrophysiological investigation of learning and memory. The honeybee olfactory pathway has been described in detail (Mobbs 1982). Higher order processing of olfactory information occurs in the mushroom body (MB) of the protocerebrum. The MB structure is found in all insects and occupies a large portion of the insect brain (Strausfeld et al. 1995). The MB has been likened to the vertebrate hippocampus and is implicated in learning and memory by anatomic, behavioral, and molecular studies (Davis 1993; Hammer and Menzel 1995). The MB receives multiple sensory inputs (Mobbs 1982), and partial or complete ablation of this structure results in defective olfactory learning (Heisenberg et al. 1985). Localized cooling of the MB induces retrograde amnesia of olfactory learning (Erber et al. 1980), and several genes that are essential for associative learning show a preferential expression in the MB (Han et al. 1992; Nighorn et al. 1991; Skoulakis et al. 1993).

The MB is a bilateral structure consisting of 340,000 Kenyon cells. The Kenyon cells are organized such that the dendrites and terminals are segregated into the calyx (input region), and the α and β lobes (output region), respectively (Mobbs 1982). The relatively small size of the Kenyon cells (4–7 μm diam) has precluded in vivo intracellular recording except in the locust MB (Laurent and Naraghi 1994). In the honeybee MB, extracellular field potentials have been recorded following presentation of external stimuli such as light and scent (Kaulen et al. 1984; Mercer and Erber 1983). More recently, an extracellular field potential consisting of pre- and postsynaptic events was recorded in vivo in the honeybee MB following electrical stimulation of the olfactory input pathway (Oleskevich et al. 1997).

Despite extensive anatomic and physiological investigations of the honeybee MB, the primary neurotransmitter mediating synaptic transmission in this region has not yet been identified. Histochemical and immunocytochemical labeling studies show that the neurotransmitter acetylcholine (ACh) and receptors for acetylcholine (AChR) are present in the MB (Kreissl and Bicker 1989; Scheidler et al. 1990). Calcium imaging studies demonstrate that cultured MB cells respond to ACh via nictinic AChR (Bicker and Kreissl 1994; Cayre et al. 1999; Goldberg et al. 1999). Glutamate immunoreactivity and excitatory responses to glutamate have been reported in the MB of different insects (Bicker et al. 1988; Cayre et al. 1999). Biogenic amines such as dopamine, serotonin, norepinephrine, and octopamine (OA) are also present in the MB (Cayre et al. 1999; Mercer et al. 1983). OA, an invertebrate monoamine and neuromodulator, has been shown to facilitate olfactory learning in the honeybee (Hammer 1993). Activation of an OA receptor specifically localized in the MB results in an increase in intracellular calcium and cyclic 3',5'-AMP (cAMP) (Han et al. 1998). The cAMP pathway has also been implicated in olfactory learning in *Drosophila*, because mutants with a defective cAMP signaling system show impaired learning (for review see Davis 1993).

In this study, we investigate the primary neurotransmitter that mediates synaptic transmission of the olfactory input pathway in the MB. A newly developed whole bee brain in vitro preparation allowed a pharmacological identification of the neurotransmitter. The viability and stability of the new preparation was confirmed by comparison of the synaptic response to a previously reported in vivo synaptic response in the MB (Oleskevich et al. 1997). The effect of cholinergic antagonists and the neuromodulator OA was investigated to determine their role in modulation of synaptic transmission in the insect olfactory pathway.

**METHODS**

**Tissue preparation**

Whole bee brains (*n* = 46) were prepared from adult foraging honeybees, *Apis mellifera*. The bees were deeply anesthetized by cooling to minimize animal suffering. A new dissection method...
allowed isolation and removal of the whole bee brain from the head capsule (Fig. 1A). The whole brain (~600 μm thick) was submerged in a recording chamber (0.5 ml), anchored with a nylon net, and continuously perfused (1.5 ml/min) with physiological saline containing (in mM) 140 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgCl₂, 1.2 NaH₂PO₄, 14 glucose, 4 Na-HCO₃, and 6 HEPES (pH 7.4; osmolarity, 322 mosM) at room temperature (24°C; Fig. 1B). After dissection, bee brain activity was allowed to recover for 20–40 min before the start of the experiment (Kirov et al. 1999).

Electrophysiology
Extracellular recordings (n = 69) were made as previously described (Oleskevich et al. 1997). Glass recording electrodes (0.5–1 MΩ) were filled with physiological saline and placed in the center of the median calyx of the MB at a depth of 50–150 μm (Fig. 1C). The recording electrode was placed in the center of the calyx, close to the Kenyon cell somata. Focal stimulation of the antennal lobe (0.1 ms; 3–9 V; 0.2 Hz) was delivered via a parylene-coated tungsten bipolar electrode (1–2 μm tip, 2 MΩ). The exposed tips of the stimulating electrode were positioned in the dorsomedial antennal lobe (~750 μm from the recording electrode) where the antenno-glomerular tracts (agt) originate (Mobbs 1982). Stimulation of the antennal lobe evoked a characteristic extracellular field response in the median calyx of the MB, presumably via activation of the antenno-glomerular tract. The characteristic response helped ensure accurate and reproducible placement of the stimulating and recording electrode. Additional experiments confirmed focal stimulation and allowed separate experiments to be performed on each side of the brain (Oleskevich et al. 1997). Paired-pulse facilitation (PPF) was evoked by two consecutive stimuli separated by 24 ms. The amplitude of the population spike or afferent volley was measured from a horizontal line joining the maximum peaks that appear before and after the response (see Fig. 1C). If the afferent volley amplitude changed by >15% during the course of the experiment, the results were not used. Data acquisition and analysis was performed with pClamp 6.0 and AxoGraph 4.0 (Axon Instruments). Extracellular responses were low-pass filtered on-line at 3 kHz. All changes in response amplitudes are expressed as a percent change from the control value. Results are expressed as means ± SE, and statistical tests of significance were determined with parametric (paired or unpaired t-tests) or nonparametric (paired sign) tests, as appropriate.

Materials
Cadmium (50–100 μM), α-bungarotoxin (0.2–0.5 μM; RBI), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 5 μM; Tocris), OA (100 μM; RBI) were added to the physiological saline and applied by bath perfusion.

RESULTS
Synaptic response in the isolated bee brain
Whole bee brains were isolated and maintained in vitro for up to 2.5 h. Extracellular field potentials were recorded in the MB calyx, following focal stimulation of the antennal lobe (Fig. 1C). As elucidated below, the field potential consisted of a presynaptic afferent fiber volley and a postsynaptic population spike superimposed on the rising phase of the field excitatory postsynaptic potential (Fig. 2A). The mean amplitudes of the afferent volley and population spike were 0.42 ± 0.04 (SE) mV and 0.65 ± 0.10 mV, respectively (n = 21). The latency from the onset of stimulation was 2.7 ± 0.1 ms for the afferent volley and 6.8 ± 0.2 ms for the population spike (n = 14). A comparison of the field potential to that previously reported in vivo (Oleskevich et al. 1997) shows that the latency from the onset of stimulation to the population spike was significantly greater in vitro (P < 0.005). The amplitude of the afferent volley and the population spike were 62 and 50% smaller, respectively, in vitro (P < 0.05; Fig. 2A).
The identities of the components of the field response were confirmed with cadmium application and paired-pulse stimulation. Bath application of cadmium (50–100 μM) transiently attenuated the amplitude of the population spike while the afferent volley amplitude was unchanged (Fig. 2B). In all animals (n = 8), cadmium significantly depressed the amplitude of the population spike by 54 ± 7% without affecting the afferent volley (7 ± 3; P < 0.01; Fig. 2C). Inhibition of the population spike by cadmium resembled in vivo inhibition by cadmium (1–10 μM; 41 ± 6%; n = 6; P < 0.05; Fig. 2C). Two consecutive stimuli induced a significant PPF of the population spike (126 ± 33%; n = 9; P < 0.005; Fig. 2D). The afferent volley did not show PPF (2 ± 5%; n = 9). PPF of the population spike (72 ± 11%; P < 0.01) but not of the afferent volley (−11 ± 5%) was also observed in vivo. Together, the inhibition by cadmium and PPF of the population spike suggest that the field potential recorded in the MB consists of a presynaptic afferent fiber volley and a postsynaptic population spike.

Synaptic response is mediated by ACh

Bath application of d-tubocurarine (30–60 μM), a competitive antagonist at the nicotinic AChR subtype, produced a transient reduction of the population spike without affecting the afferent volley (Fig. 3A). d-Tubocurarine significantly inhibited the population spike by a mean of 61 ± 6%, while the afferent volley was unchanged (−4 ± 5%; n = 9; P < 0.005). The irreversible nicotinic AChR antagonist, α-bungarotoxin (α-Bgt; 0.2–0.5 μM), was bath applied for 15–30 min to ensure maximal diffusion through the preparation. α-Bgt attenuated the population spike that showed no recovery 15–20 min after drug wash out (Fig. 3B). α-Bgt significantly attenuated the response by 62 ± 5% without significantly affecting the afferent volley (−21 ± 8%; n = 5; P < .0001). Antagonists at the glutamate N-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subtypes, d-AP5 (50 μM) and CNQX (5 μM), respectively, did not affect the amplitude of the population spike (Fig. 3C). Coapplication of d-AP5 and CNQX had no effect on the population spike (2 ± 3%; n = 7; Fig. 3D).

Modulation by OA

Bath application of the invertebrate monoamine, OA (100 μM), transiently increased the population spike without affecting the afferent volley (Fig. 3E). On average, the population spike more than doubled in the presence of OA. The mean increase of the population spike was 129 ± 68% (n = 7; P < 0.01) and −5 ± 4% for the afferent volley (n = 6; Fig. 3F). In three preparations, the enhancement of the population spike by OA was followed by a depression of both the afferent volley (−85 ± 4%) and the population spike (−93 ± 2%; data not shown).

DISCUSSION

A newly developed whole bee brain in vitro preparation allowed an investigation of the pharmacology and modulation of a synaptic response in the MB of the honeybee. The suitability of the new preparation was confirmed by comparison of the synaptic response to a response previously recorded in vivo. Inhibition by cadmium suggested an effective drug perfusion, whereas paired-pulse facilitation demonstrated a viable tissue preparation. The synaptic response was inhibited by the cholinergic receptor antagonists, d-tubocurarine and α-bungarotoxin but not by the glutamate receptor antagonists, AP5 and CNQX. OA transiently increased the synaptic response. The results suggest that the synaptic response is mediated primarily by ACh and can be modulated by OA.

Isolated whole bee brain preparation

The whole brain in vitro preparation provided access to the neural circuitry responsible for olfactory learning in the honeybee. The viability of the preparation is evident in the similarity of the synaptic response to the response recorded in vivo with regard to time course, PPF, and inhibition by cadmium.
This preparation has the advantage over the in vivo honeybee preparation in that the addition of pharmacological agents to the physiological saline ensures thorough application at an effective concentration. In the in vivo preparation, higher drug concentrations were required to evoke similar effects (Oleskevich et al. 1997). The in vitro preparation has an advantage over mammalian slice preparations and cultured honeybee MB cells, in that the brain is kept whole and the neural circuitry remains intact. A “semi-slice” preparation has been described for the blow fly brain (Brotz et al. 1995), and an in vitro preparation was recently employed for characterization of honeybee antennal lobe neurons (Kloppenburg et al. 1999). Our in vitro recordings of honeybee MB neurons helped to identify the neurotransmitter and receptor subtype, which may underlie higher-order olfactory information processing in the honeybee.

Focal stimulation in the antennal lobe evoked an extracellular field response in the MB. The field response was composed of a presynaptic afferent volley and a postsynaptic population spike. The amplitude of the afferent volley and the population spike was smaller in vitro than in vivo. The synaptic efficacy was not compromised because both the afferent volley and the population spike were similarly attenuated. The efficiency of stimulation may have been reduced due to a change in orientation of the stimulating electrodes and the antennal lobe in vitro versus in vivo. The latency from the onset of stimulation to the peak of the afferent volley and the population spike was similar to that observed in vivo, further reinforcing the presence of a mono- rather than polysynaptic response (Oleskevich et al. 1997).

The population spike showed attenuation by cadmium and PPF. PPF is generally assumed to be a synaptic phenomenon in which presynaptic residual calcium causes an increase in neurotransmitter release when a second stimulation is applied. Cadmium is a calcium channel blocker and can inhibit calcium-dependent neurotransmitter release and hence the postsynaptic response, without affecting the sodium channel-dependent presynaptic volley. The concentration of cadmium used did not fully block the calcium channels. Together, these results further support that the field potential recorded in the MB consists of a presynaptic afferent fiber volley and a postsynaptic population spike (Oleskevich et al. 1997).

**Pharmacology and modulation of the synaptic response**

The inhibition of the population spike by α-tubocurarine and α-Bgt provided the first direct physiological evidence that ACh mediates synaptic transmission between the antennal lobe and...
the MB. Specifically, the inhibition of the synaptic response by α-Bgt suggests that ACh is acting via a nicotinic AChR subtype containing the α7 subunit (Couturier et al. 1990). Nicotinic, muscarinic, and mixed nicotinic/muscarinic ACh receptors have been observed in the nervous tissue of different insects (Breer and Sattelle 1987). In the honeybee, labeling studies with acetylcholinesterase, AChR antibodies, and iodinated α-Bgt demonstrate an abundance of ACh and AChRs in the calyx of the MB, where the afferent input fibers from the antennal lobe terminate on the dendrites of the Kenyon cells (Kreissl and Bicker 1989; Mobbs 1982; Scheidler et al. 1990). These studies were unable to exclude the possibility of presynaptic nicotinic AChRs, which modulate rather than mediate synaptic transmission in vertebrate CNS (Wonnacott 1997).

There could be several explanations for the component of the population spike, which remained following α-Bgt application. An α-Bgt−insensitive subtype of nicotinic AChR has been described in insects (Benke and Breer 1989), and an incomplete α-Bgt mediated inhibition of an ACh response has been reported in the honeybee MB (Bicker and Kreissl 1994; Goldberg et al. 1999). However, this cannot account for the component remaining after d-tubocurarine application, which is known to block both the α-Bgt−sensitive and insensitive nAChRs in vertebrates (Zhang et al. 1996). A d-tubocurarine− and α-Bgt−insensitive nicotinic AChR may exist in insects, or the remaining component may result from the co-release of a secondary transmitter. There is evidence for glutamate immunoreactivity in the calyx of the locust MB (Bicker et al. 1988), and glutamate excitatory responses were observed in most cultured MB neurons in the cricket (Cayre et al. 1999). However, the lack of effect of AP5 and CNQX in the honeybee suggests that the remaining component is not glutamate acting at glutamate receptors with known mammalian synaptic receptor pharmacology.

The invertebrate monoamine, OA, increased the amplitude of the population spike. Previous studies have shown that OA can increase the extracellular field response to external olfactory stimuli (Mercer and Erber 1983). Activation of an OA receptor in the MB of Drosophila elevates intracellular calcium and stimulates cAMP (Han et al. 1998). Perhaps the activation of a cAMP signaling pathway provides a molecular basis for the ability of OA to facilitate olfactory learning in the honeybee (Hammer 1993). The cAMP pathway has been implicated in learning in Drosophila mutants, which are defective in cAMP phosphodiesterase (dnc), adenylyl cyclase (rut) or protein kinase A (DCO) and show impaired associative learning (for review see Davis 1993). The biphasic nature of the OA modulation that we observed in some animals has been reported elsewhere (Cayre et al. 1999). OA may have complex actions that include increased excitation and decreased inhibition.

Conclusion

This study provides physiological evidence that synaptic transmission of the olfactory input pathway to the MB is mediated primarily by ACh acting at the nicotinic AChR. The ACh response was enhanced by OA, an important neuromodulator for olfactory learning in the honeybee.

The author is grateful to Drs. J. D. Clements and M. V. Srinivasan for useful discussions and comments on the manuscript.

Address for reprint requests: S. Oleskevich, Division of Neuroscience, John Curtin School of Medical Research, Australian National University, P.O. Box 334, Canberra, A.C.T. 2601, Australia.

Received 1 March 1999; accepted in final form 29 April 1999.

REFERENCES


