Cultured Insect Mushroom Body Neurons Express Functional Receptors for Acetylcholine, GABA, Glutamate, Octopamine, and Dopamine

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Cayre, M., S. D. Buckingham, S. Yagodin, and D. B. Sattelle. Cultured insect mushroom body neurons express functional receptors for acetylcholine, GABA, glutamate, octopamine, and dopamine. J. Neurophysiol. 81: 1–14, 1999. Fluorescence calcium imaging with fura-2 and whole cell, patch-clamp electrophysiology was applied to cultured Kenyon cells (interneurons) isolated from the mushroom bodies of adult crickets (Acheta domesticus) to demonstrate the presence of functional neurotransmitter receptors. In all cells investigated, 5 μM acetylcholine (ACh, n = 52) evoked an increase in intracellular free calcium ([Ca2+]i). Similar effects were observed in response to 10 μM nicotine. The ACh response was insensitive to atropine (50 μM) but was reduced by mecamylamine (50 μM) and α-bungarotoxin (α-bgt, 10 μM). ACh-induced inward ion currents (n = 28, E_{ACh} = 0 mV) were also blocked by 1 μM mecamylamine and by 1 μM α-bgt. Nicotine-induced inward currents desensitized more rapidly than ACh responses. Thus functional α-bgt-sensitive nicotinic ACh receptors are abundant in all Kenyon cells tested, and their activation leads to an increase in [Ca2+]i. γ-Aminobutyric acid (GABA, 100 μM) triggered a sustained decrease in [Ca2+]i. Similar responses were seen with a GABAa agonist, muscimol (100 μM), and a GABAb agonist, 3-APPA (1 mM), suggesting that more than one type of GABA receptor can affect [Ca2+]i. This action of GABA was not observed when the extracellular KCl concentration was lowered. All cells tested (n = 26) with patch-clamp electrophysiology showed picrotoxin (PTX)-sensitive, GABA-induced (30–100 μM) currents with a chloride-sensitive reversal potential. Thus, an ionotropic PTX-sensitive GABA receptor was found on all Kenyon cells tested. Most (61%) of the 54 cells studied responded to L-glutamate (100 μM) application either with a biphasic increase in [Ca2+]i or with a single, delayed, sustained [Ca2+]i increase. Nearly all cells tested (95%, n = 19) responded to (100 μM) t-glutamate with rapidly desensitizing, inward currents that reversed at approximately −30 mV. Dopamine (100 μM) elicited either a rapid or a delayed increase in [Ca2+]i, in 63% of the 26 cells tested. The time course of these responses varied greatly among cells. Dopamine failed to elicit currents in patch-clamped cells (n = 4). A brief decrease in [Ca2+]i was induced by octopamine (100 μM) in ~54% of the cells tested (n = 35). However, when extracellular CaCl2 was lowered, octopamine triggered a substantial increase in [Ca2+]i, in 35% of the cells tested (n = 26). No octopamine-elicited currents were detected in patched-clamped cells (n = 10).

INTRODUCTION

The mushroom bodies of insects are of special interest because of the key position of these neural structures among a variety of sensory inputs and forward outputs to many areas of the brain (Fig. 1A) (Howse 1975; Menzel and Muller 1996; Strausfeld et al. 1995). In the cricket (Acheta domesticus), each mushroom body consists of a group of 50,000 clustered interneuron cell bodies in the dorsal posterior cortex and an associated neuropil that includes the projections of these intrinsic neurons (named Kenyon cells) and their synaptic contacts with afferent and efferent neurons. In the last 2 decades, insect mushroom bodies were demonstrated to show morphological plasticity even in adults. For example, Bieber and Fuldner (1979) reported changes in volume and structure of the mushroom bodies during the adult life of a coleopteran, Aleochara curvata. In the fruitfly, Drosophila melanogaster, the size of these structures varies with age, sex, and environment (Heisenberg et al. 1995; Technau 1984; Technau and Heisenberg 1982). In the honeybee, Apis mellifera, mushroom bodies undergo an internal reorganization during behavioral development, with the ratio of neuropil volume to Kenyon cell body volume increasing markedly between nursing and foraging bees (Durst et al. 1994; Fahrbach and Robinson 1996; Withers et al. 1993). A further example of adult plasticity is the recent demonstration that neuroblasts persist in mushroom bodies of certain adult orthopteran and coleopteran insects and continue to generate Kenyon cells (Cayre et al. 1994, 1996). In the cricket, Acheta domesticus, this adult neurogenesis appears to be regulated by the two morphogenetic hormones, ecdysone and juvenile hormone (Cayre et al. 1994). These data underline the remarkable plasticity of mushroom bodies, suggesting special functional roles for these structures.

Indeed, much of the latest evidence indicates that Dujardin was not entirely without justification in postulating a role for these structures as the center of ‘insect intelligence’ (Dujardin 1850). Development of Drosophila mutants with disrupted mushroom bodies, mushroom body deranged (mbd) and mushroom body miniature (mbm), implicated these structures in olfactory learning (Heisenberg et al. 1985). Block of neuronal transmission in mushroom bodies by local cooling resulted in amnesia in honeybees (Erber et al. 1980). Chemical ablation of the neuroblasts responsible for formation of mushroom bodies abolished learning in Drosophila (deBelle and Heisenberg 1994), and microinjections of these structures impaired place memory in cockroaches (Mizunami et al. 1993). Participation of mushroom bodies in learning and memory is considered to involve the adenosine 3′,5′-cyclic monophosphate (cAMP) signaling pathway because the affected genes of three Drosophila mu-
subsets of Kenyon cells (Ferveur et al. 1995; O’Dell et al. 1995; Yang et al. 1995).

However, Kenyon cell neurotransmitters and their receptors are not well understood. This is due largely to the difficulty of ascribing the immunocytochemical labeling observed to the intrinsic neurons themselves. Acetylcholine (ACh)-mediated pathways have been most fully investigated. In the honeybee, the lips of the calyces as well as the lobes and pedunculi of mushroom bodies are labeled by nicotinic ACh receptor (nAChR) antibodies (Kreissl and Bicker 1989) and by $^{[125]}\text{I}$-bgt (Scheidler et al. 1990). However, no acetylcholinesterase (AChE) activity could be detected in Kenyon cells (Kreissl and Bicker 1989). The expression of nAChRs on Kenyon cells was later confirmed by $^{[3]}\text{H}$-Octopamine and $^{[3]}\text{H}$-serotonin binding were observed in mushroom body neuropil (Brüning et al. 1987), but surprisingly this staining was not matched by octopamine and serotonin immunoreactivity (Erber et al. 1993). Immunocytochemical studies revealed that the lobes and pedunculi of the mushroom bodies were richly innervated by dopaminergic fibers (Nässel and Elekes 1992), and recently a new dopamine receptor was cloned in Drosophila and shown to be expressed preferentially in the mushroom bodies (Han et al. 1996). High levels of taurine and some L-glutamate immunoreactivity have been detected in honeybee Kenyon cell bodies and in calyces (Bicker and Kreissl 1994). $^{[3]}\text{H}$-Octopamine and $^{[3]}\text{H}$-serotonin binding were recorded in insect Kenyon cells (Bicker et al. 1985; Homberg et al. 1987; Leitch and Laurent 1996; Schäfer and Bicker 1986).

Voltage-gated ionic currents were recorded in cultured pupal honeybee (Schäfer et al. 1994) and larval Drosophila (Wright and Zhong 1995) Kenyon cells. Odor-evoked responses were recorded in locust Kenyon cells (Laurent and Naraghi 1994). To date, there is no functional evidence for amino acid neurotransmitter-evoked ion currents in Kenyon cells of any species. However, with the exception of the demonstration of ACh-induced $[^{2}]\text{Ca}^{2+}$, increases in pupal honeybee Kenyon cells (Bicker and Kreissl 1994), no functional responses to neurotransmitters or neuromodulators were recorded in insect Kenyon cells.

We developed an in vitro approach (Fig. 1B) to the study of adult Kenyon cells (Cayre et al. 1998). In this study, we used fura-2 AM calcium imaging and whole cell patch-clamp recordings of membrane currents to demonstrate functional neurotransmitter receptors for ACh, GABA, L-glutamate, octopamine, and dopamine in adult Kenyon cells of the cricket, Acheta domestica.

**METHODS**

**Animals**

Crickets (A. domestica) were maintained under a long-day photoperiod (16 h light:8 h dark), at 29°C and 55% relative humidity. They were fed wheat germ, bran, and corn oil; water was continuously available. Adults were isolated and dated from the day of emergence; 1–8 day-old females were used for dissection.
Cell preparation

Crickets (A. domesticus) were cold-anesthetized, sterilized in 70% ethanol, and rinsed with sterilized water. The brain was carefully removed from the head capsule, taking care not to tear the alimentary tract. Dissection was carried out in a Ca\(^{2+}\)- and Mg\(^{2+}\)-free Acheta saline to facilitate cell dissociation. Normal Acheta saline was of the following composition (in mM): 150 NaCl, 12 KCl, 3 MgCl\(_2\), 15 CaCl\(_2\), 10 N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid (HEPES), 40 glucose, pH 7.2, 400 mosmol. In divalent cation-free saline, the osmolarity was adjusted to 400 mosmol by addition of trehalose. The neuronal sheath was removed with forceps, thereby exposing the cell body rinds of the mushroom bodies, which were then dissected from the brain and collected in an Eppendorf tube containing culture medium. The mushroom bodies of 20 brains were collected in this way. The pooled mushroom bodies were rinsed with culture medium and dissociated by gentle trituration through siliconized, fire-polished Pasteur pipettes. No enzymes were used, as those tested in preliminary trials (collagenase and trypsin) were found to dramatically decrease cell viability. A 100-μl aliquot of the cell suspension (containing ~8,000 cells) was gently poured onto a glass coverslip covered with medium in a culture dish (Falcon 1006) coated with poly-D-lysine (0.005%). Cells were allowed to adhere for 1 h, and then covered culture dishes were stored at 30°C in a humidified culture chamber.

Culture medium

L-15 medium (GIBCO) was used, supplemented with 0.4 g glucose and 2 ml penicillin-streptomycin (5,000 UI, 5,000 μg/ml; GIBCO) per 100 ml medium (pH 7.0, 400 mosmol).

Calcium imaging

Experiments were performed on freshly dissociated cells (i.e., 1–24 h after dissociation). To remove serum, cells were washed gently in normal Acheta saline (for composition, see CELL PREPARATION) or, where indicated in the text, with modified saline (in mM: 129 NaCl, 8.6 KCl, 8.5 MgCl\(_2\), 2 CaCl\(_2\), 34 glucose, and 15 HEPES). Cells were then loaded with the calcium-sensitive dye fura-2 AM (4 μM) in the same saline for 40 min at room temperature in the dark. After loading, cells were thoroughly rinsed in normal saline to facilitate cell dissociation. Normal Acheta saline (for composition, see CELL PREPARATION) was used to wash out the experiment to allow for correction by pixel-by-pixel subtraction of background fluorescence. The ratio of emitted fluorescence at the two excitation wavelengths (340 and 380 nm) was selected by means of a computer-controlled rotating filter wheel located between a xenon lamp and the microscope. Emitted light at 510 nm (40 nm half-bandwidth) was passed to an image-intensifying charge-coupled device camera (Photogenic Science, UK). The resulting 256 × 256 pixel images at each wavelength were averaged in real time, typically 8 frames per image, and digitized at 8 bits accuracy to yield 256 gray levels. Background images (at 340 and 380 nm), taken from an area of the coverslip containing no cells, were captured at the start of each experiment to allow for correction by pixel-by-pixel subtraction of background fluorescence. The ratio of emitted fluorescence at the two excitation wavelengths was calculated for each frame after background subtraction.

To produce a continuous trace of mean ratio against time, an area around the cell was graphically defined with a light pen and the mean ratio level within the area computed. This could be achieved for up to eight cells at one time. The computed ratio value was calculated pixel by pixel. The mean of all values in the pixel set was taken as the mean ratio. Individual data sets were exported to ASCII files for subsequent analysis.

Putative ligands were applied to the cells on coverslips for 15 s with bolus delivery into a 200-μl bath volume. A rapid aspirator was used to remove test solutions and to maintain a constant volume. At least 5 ml normal Acheta saline was used to wash out the coverslip after each drug application.

Unless otherwise indicated, data are expressed as ratios of the emitted fluorescence at the two excitation wavelengths (340 and 380 nm). In histograms, data are expressed as R/R\(_0\) × 100, where R refers to the fluorescence ratio at the peak of the response and R\(_0\) refers to the fluorescence ratio of the same cell before drug application.

![FIG. 2. Effects of depolarization by 50 mM KCl on [Ca\(^{2+}\)] of dissociated Kenyon cells. A: representative trace from 2 single cells illustrating [Ca\(^{2+}\)] response. The black thick trace under each time scale indicates the time and duration of drug application. B: histogram showing quantitative and statistical data for the KCl-induced [Ca\(^{2+}\)] response. Column represents mean value at the peak of effect ± SE expressed as a percentage of the fluorescence ratio just before KCl application (Control, 100%). Number above column indicates sample size.](http://jn.physiology.org/)

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Basal $[\text{Ca}^{2+}]$, was estimated according to the following formula (Grynkiewicz et al. 1985)

$$[\text{Ca}^{2+}] = \frac{K_d (R - R_{\text{max}})}{(R_{\text{max}} - R)} \times \frac{S_{\text{I}}}{S_{\text{O}}}$$

where $K_d$ is the dissociation constant for fura-2/AM/Ca$^{2+}$ (225 nM); $R_{\text{max}}$ and $R_{\text{min}}$ are the fluorescence ratio values at 0 and saturating $[\text{Ca}^{2+}]$, respectively, and $S_{\text{I}}$/$S_{\text{O}}$ is the ratio of fluorescence values for free and bound fura-2 AM at the higher of the two wavelengths (380 nm). Saturating $[\text{Ca}^{2+}]$, was obtained by permeabilizing the cells with 2 μM ionomycin in the presence of 2–15 mM $[\text{Ca}^{2+}]_o$ (respectively for modified saline and standard saline) and 0 $[\text{Ca}^{2+}]_o$ by rinsing the cells with a Ca$^{2+}$-free saline containing ethylene glycol-bis(β-aminooethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 1–20 mM).

**Electrophysiology**

Experiments were performed on cells 1–5 days after dissociation. Cells cultured on glass coverslips were washed twice in normal Acheta saline to remove serum and then incubated for 1 min in saline containing 0.2% Trypan blue. After two rinses in saline, the plates were transferred to a petri dish containing normal Acheta saline. Cell membrane currents were measured with tight seal, whole cell, patch-clamp recording techniques (Hamill et al. 1981) Glass pipettes were pulled from borosilicate glass (Clark Electromedical, U.K.) on a vertical pipette puller (Narishige PP-83, Japan) and had a resistance of 7–10 MΩ when filled with a solution containing (in mM) 194 potassium gluconate, 20 KCl, 4 MgCl₂, and 10 HEPES (adjusted to pH 7.2 with 1N KOH), except where indicated in the text.

Cells 8–15 μm in diameter, the most abundant in the cultures (see Cayre et al. 1998), were selected for electrophysiology. Viable cells (those resistant to staining with Trypan blue) readily formed stable, gigohm seals on contact with patch pipettes, and the whole cell recording mode was routinely achieved. Whole cell recordings were achieved by applying gentle suction to the pipette after the formation of a stable gigohm seal. Currents were recorded under voltage clamp with a List EPC7 patch-clamp amplifier (List-Medical, Germany). Digitized currents were stored on a Viglen IBM 486DX personal computer and were analyzed by means of the pClamp 6.03 suite of data analysis and acquisition software (Axon Instruments), which was also used to generate voltage-clamp protocols. Cells were clamped at a holding potential of −60 mV, and the series resistance (the sum of all the resistances between the amplifier headstage and the interior of the cell) was compensated by ≥80% by a feed-forward circuit incorporated in the patch-clamp amplifier.

During electrophysiological experiments, cells were continuously perfused with normal Acheta saline applied through a U-tube placed 1–2 mm from the cell. Ligands were either bath applied or applied by pressure-ejection from a patch pipette filled with the ligand (100 μM in physiological saline of the same composition as the perfusing saline) placed <50 μm from the cell. In the case of bath application, the perfusing saline was substituted for one containing the ligand diluted to the required concentration from a stock solution of 10 mM in dimethyl sulfoxide (picrotoxinin) or normal Acheta saline (all other ligands). The reversal potentials of GABA-induced currents were measured by applying a ramp waveform on the holding potential from −100 mV to +40 mV before, and then during, a 3- to 10-s pressure application of the ligand. The ramp protocol consisted of holding the cell at −100 mV for 100 ms and then changing the clamped potential at a uniform rate to +40 mV over a period of 700 ms. The ramp currents measured before the drug application were subtracted from those measured during the application to give the ligand-induced currents, which were then plotted against the clamped membrane potential. The reversal potentials of ACh- and l-glutamate-induced currents could not be measured in this way because they desensitized rapidly. Instead, current–voltage relationships were determined by measuring the peak amplitude of the responses to 200- to 300-ms pressure pulses of the test compound, repeated over a range of steady holding potentials.

In all experiments, sufficient time was allowed between successive exposures to ligands to prevent desensitization, and cells were exposed to no more than one antagonist. The effect of repeated applications of the same neurotransmitter on the same cells was tested and it was found that the responses could be repeatedly obtained when a resting time of 5 min was interposed between applications.

**Data analysis**

All data points represent the mean of at least three separate experiments performed on separate cells, and error bars represent ± 1 SE. Significant differences ($P < 0.05$) were evaluated with the nonparametric Mann Whitney U test.

**Chemicals**

Leibovitz L-15 medium and penicillin-streptomycin were purchased from Gibco, and fura-2AM was purchased from Molecular Probes. All other chemicals were purchased from Sigma, except for 3-APPA, which was purchased from Research Biochemicals International.

**RESULTS**

The resting level of $[\text{Ca}^{2+}]$ in dissociated fura-2 AM-loaded adult Kenyon cells was estimated to be 126 ± 22 nM (n = 8) in standard saline and 60 ± 17 nM (n = 8) in modified (low calcium) saline. Application of saline containing elevated K⁺ (50 mM) to depolarize the cells triggered only a small increase (26 ± 4%, n = 10, Fig. 2) in $[\text{Ca}^{2+}]$, in cell somata in 66% of cells tested. In contrast, an experiment performed on clusters of Kenyon cells with a bundle of processes that had resisted desensitization showed more pronounced responses to elevated K⁺ in neurites compared with cell bodies (data not shown), suggesting a differential distribution of voltage-gated Ca$^{2+}$ channels in these neurons.

ACh evoked a rapid increase in fluorescence ratio within 1–5 s of application, indicating a strong rise in $[\text{Ca}^{2+}]$, (Fig. 3B). ACh (0.1 μM) was sufficient to increase the fluorescence ratio (Fig. 3A) to 126 ± 5% of basal values (n = 11, $P = 0.016$). The response reached 144 ± 11% of basal values when ACh 1 μM was applied (n = 11, $P < 0.001$). However, more reproducible signals of higher am-
amplitude were detected at 5 μM ACh, which was therefore the concentration used in most subsequent experiments. All cells tested responded to ACh (n = 52). At the peak of the response, which was always 10–20 s after the beginning of drug application, the fluorescence ratio reached a maximum of 219 ± 16% of basal values (Fig. 3E; P < 0.001). On rebathing in normal saline, [Ca^{2+}]_i recovery occurred after >80 s and was sometimes incomplete at the end of the recording time (≤150 s, Fig. 3B). Nicotine (10 μM) mimicked the effects of 5 μM ACh application (Fig. 3C), resulting in a strong increase in the fluorescence ratio (287 ± 13%; P < 0.001; Fig. 3E), whereas the muscarinic agonist oxotremorine (100 μM) only very slightly enhanced the fluorescence ratio (114 ± 4%; P = 0.028, Fig. 3, D and E). Atropine (≤50 μM) did not reduce the amplitude of the ACh response (Fig. 3F), whereas the nicotinic antagonists mecamylamine (50 μM) and α-bungarotoxin (α-bgt, 10 μM) strongly reduced the ACh-induced increase in fluorescence ratio to ~15 and 11%, respectively, of control values (p < 0.001; Fig. 3, G and H). When extracellular Ca^{2+} was removed from the saline (EGTA 1 mM), only 3 of 15 cells responded to ACh (data not shown). All 28 cells tested under whole cell voltage clamp responded to pressure-applied ACh (100 μM) with inward currents that reversed at approximately −5 mV, n = 4 (Fig. 4D). Responses to repeated applications of 100 μM ACh (1 min −1) showed no evidence of rundown. No responses to ACh were observed when the cells were bathed in a saline containing 1 μM α-bgt, and bath application of 1 μM mecamylamine reduced the peak amplitude of ACh-induced currents by 82 ± 13% (n = 3, Fig. 4B). Bath-applied atropine (50 μM) reduced the peak amplitude of ACh-induced currents by 57 ± 15% (n = 3, Fig. 4C), whereas 1 μM atropine resulted in a 16 ± 19% (n = 4) reduction in the amplitude of the ACh response. Nicotine (100 μM) also induced inward currents at a clamped holding potential of −60 mV (Fig. 4A), but the responses to nicotine desensitized much more rapidly than those to ACh.

GABA (100 μM) application resulted in a decrease in [Ca^{2+}]_i (P < 0.001, Fig. 5). As was the case for ACh, the effect occurred within seconds of drug application. The maximal decrease in fluorescence ratio in normal saline (down to 73 ± 3% of resting values; Fig. 5E) was reached 40–60 s after application and was sustained even after 60 s of wash (Fig. 5A). Interestingly, cells with low basal [Ca^{2+}]_i (<100 nM) were insensitive to GABA. Application of muscimol (100 μM), a vertebrate GABA_α receptor agonist, induced a similar decrease when tested in normal saline (72 ± 4% n = 13; Fig. 5B); 3-APP (1 mM), an agonist of vertebrate and insect (Bai and Sattelle 1995) GABA_β receptors, also triggered a reduction of the fluorescence ratio (Fig. 5, C and E). This suggests that the GABA-induced decrease in [Ca^{2+}]_i results from activation of a mixed popula-

GABA receptor subtypes. As the GABA-induced decrease in fluorescence ratio seemed to depend on the initial fluorescence ratio, the effect of lowering extracellular [Ca^{2+}]_i on the GABA response was tested. When modified saline containing lower [Ca^{2+}]_i was used (2 cf. 15 mM), no cells responded to GABA application (n = 23; Fig. 5, D and F). Under whole cell patch-clamp conditions in normal saline, all cells tested (n = 26) responded to bath-applied GABA (10–100 μM) with rapidly desensitizing, dose-dependent, inward currents at a clamped membrane potential of −60 mV (Fig. 6A). Pressure-applied GABA evoked similar responses, which increased in amplitude as the duration of the pressure pulse was increased (Fig. 6B). GABA-induced currents evoked by pressure application of GABA (100 μM) were rapidly blocked by bath application of picrotxin (100 μM, Fig. 6C). The reversal potential of GABA-induced currents in normal saline (E_GABA) was estimated to be −35 ± 7 mV, n = 4. In cells bathed in saline in which the sodium chloride was replaced with equimolar potassium gluconate and normal extracellular saline used, E_GABA was −61 ± 11 mV, n = 4 (Fig. 5D).

[Ca^{2+}]_i responses to L-glutamate (100 μM) application differed from cell to cell; of the 61% of cells that responded to L-glutamate, 52% showed a delayed sustained increase in fluorescence ratio (178 ± 7%; P < 0.001, Fig. 7B) 15–40 s after neurotransmitter application, 30% showed a biphasic response composed of an immediate and transitory peak (148 ± 10%; P = 0.003) followed by the delayed and long-lasting increase in fluorescence ratio, and the remaining 18% exhibited an immediate, but sustained, increase (Fig. 7A). Nearly all cells (95%, n = 19) tested responded to pressure application of L-glutamate with inward currents at a clamped membrane potential of −60 mV with an estimated reversal potential of −32 ± 1.4 mV, n = 3 (Fig. 7, C and D). Responses to more prolonged application of the ligand showed pronounced, rapid desensitization, which was less marked when the current was outwardly directed (Fig. 7C). When GABA (100 μM) was pressure applied to a cell during a prolonged (>15 s) exposure to L-glutamate (100 μM), a large response to GABA was still seen, although the response to L-glutamate had completely desensitized (Fig. 7E).

Dopamine (100 μM) application on dissociated Kenyon cells induced an increase in the fluorescence ratio to 153 ± 7% of basal levels (Fig. 8B) in 63% of the 41 cells tested (P < 0.001). The rest of the cells showed no response to dopamine. The time course of the response differed from cell to cell; one-third of the cells exhibited an immediate response (n = 9), and the remainder (n = 17) showed a 20- to 60-s delayed response. The duration of the [Ca^{2+}]_i increase also varied between the cells (Fig. 8A). None of the cells tested under whole cell voltage clamp responded to dopamine at a holding potential of −60 mV (n = 4).

Octopamine (100 μM) application induced in 54% of the cells tested (n = 35) a significant (P = 0.024) decrease in fluorescence ratio to 78 ± 3% of control values (Fig. 8B). The effect appeared during drug application and returned to resting levels after ~50 s wash in normal saline (Fig. 9A). When the cells were assayed with the modified (low Ca^{2+}) saline, octopamine never induced any decrease in the fluorescence ratio but on the contrary triggered an increase (to 152 ± 11%; Fig. 9, C and D) in 35% of the cells tested (n = 26). None of the cells tested under whole cell voltage-clamp responded to octopamine at a holding potential of −60 mV (n = 10).

Applications of serotonin or taurine (both at 100 μM)
FIG. 4. ACh- and nicotine-induced currents recorded from dissociated Kenyon cells recorded under whole cell patch clamp. Stable responses without rundown could be elicited from cells for ≥20 min. ACh and nicotine were pressure applied from a pipette placed close (<50 μm) to the cell, whereas putative antagonists were applied in the perfusing saline. The action of α-bgt often required a longer time than the cells could reproducibly be held under whole cell patch clamp. A: both nicotine and ACh evoked desensitizing inward currents (at 0 mV), but the response to nicotine desensitized much more rapidly than that to ACh. B, left: current response to ACh (Control) is partially blocked by a 3-min perfusion of mecamylamine (1 μM). B, right: no current responses were observed in cells perfused in saline containing 1 μM α-bgt. C: current response to ACh (Control, left) is partially reduced by a 3-min perfusion of 50 μM atropine (right). D: current–voltage plot of the ACh-induced responses indicates that the current reverses at approximately –5 mV. This plot was determined by measuring the amplitude of responses to brief (300 ms) applications of ACh from a pressure pipette placed close to the cell. This was repeated over a range of steady holding potentials and the amplitude of the responses plotted against the membrane potential.

This study demonstrates for the first time the presence of five different functional neuroreceptors on the membranes of dissociated Kenyon cells from an adult insect (A. domesticus). Resting values for [Ca^{2+}]i in adult dissociated Kenyon cells are higher than those reported in other adult insect neurons [126 ± 22 nM compared with 95 ± 30 nM for cockroach DUM neurons in culture (Grolleau et al. 1996) and 46 nM for thoracic cockroach neurons in cultures (Howes et al. 1991)]. This high [Ca^{2+}]i is probably due in part to the high calcium and potassium concentrations in the standard Acheta saline (15 mM CaCl₂ and 12 mM KCl), as it decreases to 60 ± 17 nM when cells are maintained in modified low-calcium saline.

ACh application elicited responses in all the cells tested in both calcium imaging and electrophysiology experiments. It is thus likely that most Kenyon cells express ACh receptors. Specific agonists and antagonists of nicotinic or muscarinic receptors were used to assess the contribution of receptor subtypes to the ACh-induced responses. Neither the muscarinic antagonist atropine (≤50 μM) nor the muscarinic agonist oxotremorine (100 μM) were very effective on the...
FIG. 6. Electrophysiological responses to GABA recorded from adult, dissociated Kenyon cells. A: pressure-applied GABA evokes dose-dependent, rapidly desensitizing inward currents. Traces of the responses of one cell to $10^{-3}$ M, $3 \times 10^{-3}$ M, and $10^{-2}$ M GABA are shown and are typical of 6–10 experiments at each concentration on separate cells. Responses to repeated applications of $10^{-4}$ M GABA were of constant amplitude. B: increasing the duration of the pressure pulse results in an increase in the amplitude of the responses. C: response to GABA (100 $\mu$M) is blocked by the bath application of 100 $\mu$M picrotoxinin. D: current-voltage plot, measured by applying ramp functions to the holding potential in the absence and presence of GABA, indicates that the reversal potential is approximately $-35$ mV in normal saline (middle curve). The curve to the left was obtained with most of the intracellular chloride ions replaced with gluconate ions, and the curve to the right was obtained with most of the extracellular chloride was replaced with gluconate. To obtain these recordings, cells were clamped at $-100$ mV for 100 ms and the holding potential then changed to $+40$ mV at a constant rate for 700 ms. The traces shown are each the averages of 3 original current recordings from separate cells. Inset: current responses to pressure-applied GABA recorded at $-60$, $-30$, and $+30$ mV (bottom trace to top trace).

ACh responses recorded with calcium imaging, although such concentrations of atropine (50 $\mu$M) partially blocked ACh-induced currents recorded with whole cell patch clamp. In whole cell patch-clamp experiments, the partial block of the ACh response by 50 $\mu$M atropine did not alter the time course of the response, suggesting that the partial block by this relatively high concentration of atropine was due to a direct action on nAChRs. Both nicotinic antagonists investigated (mecamylamine and $\alpha$-bgt) strongly inhibited ACh-induced increases in $[Ca^{2+}]_i$, and ACh-induced currents, suggesting that ACh activates only $\alpha$-bgt sensitive, nicotinic receptors. It is therefore likely that ACh and nicotine act on the same receptors but evoke responses with differing kinetics and that these nAChRs are partially sensitive [similar to the nAChRs present on identified motor neurons (David and Sattelle 1984) and modulatory neurons (Lapied et al. 1990) of the cockroach to atropine]. Results reported here are in agreement with the findings of Bicker and Kreissl (1994) obtained in the pupal honeybee (Apis mellifera) and with results of immunohistochemical and radioligand binding studies in the adult honeybee showing afferent cholinergic projections from olfactory neurons into the lip of the mush-
FIG. 7. Effects of L-glutamate on \([\text{Ca}^{2+}]_{i}\) and transmembrane currents of dissociated Kenyon cells. 

A: representative traces of \([\text{Ca}^{2+}]_{i}\) transient from single cells showing different kinds of responses. The black solid bar under each x-axis indicates the time and duration of drug application.

B: histogram summarizing data for the L-glutamate–induced \([\text{Ca}^{2+}]_{i}\) response. Column represents mean value at the peak of effect ± SE. Number above column indicates sample size.

C: L-glutamate–induced rapidly desensitizing currents in Kenyon cells. Voltage dependence and desensitization of the current were sensitive to the direction of the current.

D: current–voltage plot of the peak L-glutamate–induced currents reveals a reversal potential of approximately \(-30 \text{ mV}\). Inset: current responses to pressure-applied (1 s) L-glutamate (100 \(\mu\text{M}\)) at the clamped membrane potentials \((\text{mV})\) indicated at the right of each trace. Scale bars: 200 pA, 200 ms.

E: responses to L-glutamate and GABA do not cross-desensitize. Repeated pressure applications of 100 \(\mu\text{M}\) GABA (arrows) evoke constant-amplitude current responses. Bath application of 100 \(\mu\text{M}\) L-glutamate (bar) evokes a rapidly desensitizing current but does not significantly diminish the current responses to pressure-applied GABA.

Changes in \([\text{Ca}^{2+}]_{i}\) and transmembrane currents induced by GABA application indicate that dissociated Kenyon cells possess functional GABA receptors. The significance of the neurotransmitter-induced decrease in \([\text{Ca}^{2+}]_{i}\) is an unexpected result, and the mechanisms involved remain unclear. The results obtained with the modified saline (i.e., no GABA–induced \([\text{Ca}^{2+}]_{i}\), decrease with lower extracellular \(\text{CaCl}_2\) and KCl) suggest that the high levels of calcium and potassium ions present in standard saline may depolarize the cells, which are then repolarized by GABA application, so reducing calcium influx through voltage-gated ion channels. Both GABA_A and GABA_B agonists (muscimol and 3-APPA, respectively) mimicked the effects of GABA on \([\text{Ca}^{2+}]_{i}\), suggesting a possible participation of both ionotropic and metabotropic receptors in the response. Patch-clamp experiments point to a role for chloride ions in mediating GABA-induced currents and their complete block by picrotoxinin, suggests that the membrane current response is mediated in large part by GABA-gated chloride channels similar to those described for other insect neurons (Sattelle 1990). The reversal potential of the GABA-induced current varies with the chloride equilibrium potential \((E_{\text{Cl}^-})\) but departs from \(E_{\text{Cl}^-}\), suggesting that other ionic species may also be in-
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![Graph A](image)

**FIG. 8.** Effects of dopamine on [Ca\(^{2+}\)]\(_i\) of dissociated Kenyon cells. A: representative traces of [Ca\(^{2+}\)]\(_i\) transient from 4 single cells illustrating 2 different kinetics for the [Ca\(^{2+}\)]\(_i\) response. The two stippled traces illustrate the early response, and the two plain traces the delayed response to dopamine. The black solid bar under x-axis indicates the time and duration of drug application. B: histogram summarizing data for the dopamine-induced [Ca\(^{2+}\)]\(_i\) response. Column represents mean value at the peak of effect ± SE. Number above column indicates sample size.

Further experiments are needed to test for a role for GABA\(_B\) receptors on Kenyon cells; it may be that the whole cell recording configuration involved the dialytic removal of intracellular components of signaling pathways necessary for indirect gating of currents by metabotropic GABA\(_B\) receptors.

This is the first direct demonstration of functional GABA receptors in Kenyon cells and is of interest in the light of recent immunohistochemical studies showing GABA-containing extrinsic synaptic terminals in the calyces and \(\alpha\) and \(\beta\) lobes of the mushroom bodies (Bicker et al. 1985; Leitch and Laurent 1996; Schäfer and Bicker 1986). These observations are also consistent with strong immunohistochemical staining found in the mushroom body neuropil of cricket (Strambi et al. 1998), *Drosophila* (Harrison et al. 1996), and *Calliphora* (Brotz et al. 1997).

The existence of functional excitatory amino acid L-glutamate receptors is strongly suggested by the increase in [Ca\(^{2+}\)]\(_i\), seen in the majority of the cells tested in response to this neurotransmitter. L-Glutamate-induced [Ca\(^{2+}\)]\(_i\) responses consisted either of a transient increase in [Ca\(^{2+}\)]\(_i\), followed by a delayed longer lasting increase, or of this latter type of response alone, suggesting functional differences among dissociated Kenyon cells. However, the extremely rapid onset and desensitization of L-glutamate–induced currents indicates that these currents are attributable to ionotropic receptors. The reversal potential of the L-glutamate–induced current (\(-30\) mV) differs from the predicted equilibrium potential for chloride, potassium, or sodium ions (\(-49, -75,\) and \(>250\) mV, respectively). Thus it seems likely that the L-glutamate–induced currents are carried by more than one ionic species. Furthermore, the possible involvement of more than one subtype of L-glutamate receptor in these responses cannot be excluded. Bicker et al. (1988) suggested that a population of Kenyon cells are glutamatergic, raising the possibility that L-glutamate may be a transmitter between certain Kenyon cells and/or some Kenyon cells both release and respond to L-glutamate.

We describe here, for the first time, functional dopamine and octopamine receptors on Kenyon cells. Our findings are in good agreement with Schäfer and Rehder (1989), who reported dopamine-like immunoreactivity of extrinsic origin in the mushroom bodies of the honeybee and with the very recent results of Blenau et al. (1998) demonstrating the expression of dopamine D1 receptor mRNA in the honeybee Kenyon cells. The responses we observe to both these neurotransmitters are most probably due to metabotropic receptors, as suggested by the relatively long delay between drug application and [Ca\(^{2+}\)]\(_i\) increase, particularly marked for dopamine, and by the absence of detectable induced currents under whole cell patch clamp. Octopamine receptors are widely distributed in the insect nervous system, and their roles are often compared with adrenergic receptors of vertebrates, involving activation of adenylate cyclase (Evans 1985). Furthermore, dopamine and octopamine were implicated in several behavioral modulations, particularly in olfactory learning and odor conditioning (Bicker and Menzel 1989; Erber et al. 1993; Mercer and Erber 1983), in which mushroom bodies are known to play a crucial role. Recently, a new dopamine receptor (DAMB) positively linked to cAMP was identified in *Drosophila*, highly abundant in mushroom bodies, in a pattern coincident with the rutabaga-encoded adenylate cyclase (Han et al. 1996). Furthermore, dopamine, octopamine, and serotonin have been shown to increase PKAII activity in cultured Kenyon cells of the honeybee (Müller 1997), and octopamine-like immunoreactivity was demonstrated in the calyces and \(\alpha\) lobes of honeybee mushroom bodies (Kreissl et al. 1994). In the locust (*Schistocerca gregaria*), one subtype of octopamine receptor appears to mediate its action via an increased level of [Ca\(^{2+}\)]\(_i\) (Evans 1984). In our study, however, we observe either a depression or an elevation of [Ca\(^{2+}\)]\(_i\) in dissociated Kenyon cells in response to octopamine, depending on the composition of the extracellular saline. Our results with GABA and octopamine suggest that the modified saline with lower CaCl\(_2\) and KCl is better adapted to such studies. We therefore suggest that the standard *Acheta* saline, which is routinely used for dissection, biochemical, and histological studies, and which originated from a paper on cockroaches (Lococo...
FIG. 9. Effects of octopamine on $[\text{Ca}^{2+}]_i$ of dissociated Kenyon cells. A and C: representative traces from single cells maintained in normal (A) or modified (C) saline. The black solid bar under each x-axis indicates the time and duration of drug application. B and D: histograms summarizing data for the octopamine-induced $[\text{Ca}^{2+}]_i$ response, in normal (B) and modified (D) saline. Column represents mean value at the peak of effect ± SE. Number above column indicates sample size.

et al. 1986), may not be optimal for all physiological experiments.

Although we did not detect any calcium response to serotonin and taurine (100 μM), we cannot rule out the presence of functional neuroreceptors for these putative primary signaling molecules on Kenyon cell membranes.

It is of interest to note that, for three of five functional neurotransmitter receptors detected in dissociated Kenyon cells (L-glutamate, dopamine, and octopamine), not all the cells were responsive. This points to a heterogeneity in the population of Kenyon cells with respect to neurotransmitter receptor expression, suggesting in turn heterogeneity of function.

Until recently, studies on the mushroom body interneurons, the Kenyon cells, were limited by difficulties of access. The development of an in vitro model permits the application of approaches such as patch-clamp electrophysiology and $[\text{Ca}^{2+}]$, imaging. The discovery of the different neurotransmitter receptors involved in mushroom body physiology is a first step toward a better understanding of the complex functions of these brain structures implicated in learning and memory.
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