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Modulatory action of acetylcholine on the Na^+-dependent action potentials in Kenyon cells isolated from the mushroom body of the cricket brain

E. Terazima, M. Yoshino *

Department of Biology, Tokyo Gakugei University, Koganei-shi, Tokyo 184-8501, Japan

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ABSTRACT

Kenyon cells, intrinsic neurons of the insect mushroom body, have been assumed to be a site of conditioning stimulus (CS) and unconditioned stimulus (US) association in olfactory learning and memory. Acetylcholine (ACh) has been implicated to be a neurotransmitter mediating CS reception in Kenyon cells, causing rapid membrane depolarization via nicotinic ACh receptors. However, the long-term effects of ACh on the membrane excitability of Kenyon cells are not fully understood. In this study, we examined the effects of ACh on Na^+ dependent action potentials (Na^+ spikes) elicited by depolarizing current injection and on net membrane currents under the voltage clamp condition in Kenyon cells isolated from the mushroom body of the cricket *Gryllus bimaculatus*. Current-clamp studies using amphotericin B perforated-patch recordings showed that freshly dispersed cricket Kenyon cells could produce repetitive Na^+ spikes in response to prolonged depolarizing current injection. Bath application of ACh increased both the instantaneous frequency and the amplitudes of Na^+ spikes. This excitatory action of ACh on Kenyon cells is attenuated by the pre-treatment of the cells with the muscarinic receptor antagonists, atropine and scopolamine, but not by the nicotinic receptor antagonist mecamylamine. Voltage-clamp studies further showed that bath application of ACh caused an increase in net inward currents that are sensitive to TTX, whereas outward currents were decreased by this treatment. These results indicate that in order to mediate CS, ACh may modulate the firing properties of Na^+ spikes of Kenyon cells through muscarinic receptor activation, thus increasing Na conductance and decreasing K conductance.

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1. Introduction

Kenyon cells are intrinsic neurons of the insect mushroom body, which have been assumed to be a site of conditioning stimulus (CS) and unconditioning stimulus (US) association in olfactory learning and memory (Hammer and Menzel, 1998; Dubnau et al., 2001; McGuire et al., 2001; Heisenberg, 2003; Schwaerzel et al., 2003). Acetylcholine (ACh) has been implicated as a neurotransmitter that mediates CS in Kenyon cells with a rapid membrane depolarization via an activation of postsynaptic ACh receptors. The electrical and pharmacological properties of insect nicotinic ACh receptors (nAChRs) have been extensively studied (Benson, 1992; Albert and Lingle, 1993; Buckingham et al., 1997; Eastham et al., 1998; Hermsen et al., 1998; van den Beukel et al., 1998; Cayre et al., 1999; Goldberg et al., 1999; Armengaud et al., 2001; Vermehrena et al., 2001; Déglise et al., 2002; Jackson et al., 2002; Fickbohm and Trimmer, 2003; Thany et al., 2003; Wüstenberg et al., 2004; Campusano et al., 2007). The inward currents passing through nAChRs have been suggested to mediate fast excitatory synaptic transmission in mushroom body Kenyon cells (Su and O'Dowd, 2003; Gu and O'Dowd, 2006). Recent Ca^{2+} imaging studies have shown that ACh increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via an activation of nAChRs in insect neurons (Benson, 1992; Bicker and Kreissl, 1994; Bicker, 1996; Buckingham et al., 1997; Cayre et al., 1999; Oertner et al., 2001; Yu et al., 2003; Campusano et al., 2007). ACh-induced increase in $[Ca^{2+}]_i$ has been considered to induce persistent changes in behavioral activity that could contribute to neuronal plasticity (Campusano et al., 2007).

ACh has also been shown to exert effects via muscarinic AChRs (mAChRs) in the insect central nervous system. There have been extensive pharmacological studies suggesting the presence of mAChR subtypes in insects (Breer and Sattele, 1987; Hannan and Hall, 1993; Trimmer, 1994, 1995). The expression of a cloned *Drosophila* mAChR in a stable *Drosophila* cell line has also been demonstrated (Millar et al., 1995). Trimmer and Weeks (1993) have suggested that mAChRs may play a role in modulating the excitabilities of the proleg retractor motoneurons. The involvement of mAChRs in behavior, olfactory learning, and memory recall (Cano-Lozano and Gauthier, 1998; Gauthier et al., 1994) and in the

* Corresponding author. Tel.: +81 423 297 521; fax: +81 423 297 521.
E-mail address: myoshi@u-gakugei.ac.jp (M. Yoshino).

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modulation of the rhythmic motor output or feeding pattern generator have also been described (Chrachri and Clarac, 1987; Gorczyca et al., 1991; Elson and Selverston, 1992).

Until recently, however, the long-term effects of ACh on the membrane excitabilities of mushroom body Kenyon cells are not fully understood. The present study, therefore, was designed to determine whether ACh signaling is involved in the long-term effects of membrane excitabilities in Kenyon cells. For this purpose, we examined the effects of ACh on repetitive Na^+ spikes elicited by prolonged depolarizing current injection in Kenyon cells isolated from the mushroom body of the cricket *Gryllus bimaculatus* and determined the underlying ionic mechanisms and receptor subtypes involved. Our data indicate that ACh is able to modulate the firing properties of Na^+ spikes in Kenyon cells via increasing tetrodotoxin sensitive Na conductance, and by decreasing K conductance through muscarinic receptor activation.

2. Materials and methods

2.1. Animals

Experiments were carried out on adult male crickets, *G. bimaculatus*, which were maintained in a colony in the Department of Biology at 25–30 °C with a relative humidity of 65–85% under a 12:12 h light–dark photoperiod. Crickets were fed on an artificial insect diet (Oriental Yeast) and supplied with water.

2.2. Kenyon cell isolation

Adult male crickets were anesthetized using CO_2 before dissection. The brain was carefully removed from the head capsule taking care not tear the alimentary tract. The mushroom bodies were then dissected out of the brain and placed in a silicone chamber (volume of 3 ml) filled with Ca^{2+}-free normal saline and incubated for 15 min. The mushroom bodies were then transferred to the vial tube containing dissociation solution (Sumitomo nerve-cell culture medium, Sumitomo Bakelite). The isolated mushroom bodies were incubated for this culture medium for 30 min at 25 °C. After incubation, the pooled mushroom bodies were rinsed with normal saline and dissociated by gentle trituration through a fire-polished pipette with an inner diameter of about 100 μm.

2.3. Perforated-patch clamp recording

Patch pipettes were pulled from capillary tubes (G-1.5, Narishige. CO., LTD) with a two stage pipette puller (PC-10, Narishige Co., LTD) and had a tip resistance of approximately 5 MΩ when filled with the appropriate solution for each experimental purpose. Freshly dispersed cells were allowed to settle on the flat-glass bottom of a silicone chamber mounted on the stage of an inverted microscope (I×70, Olympus), and the patch electrode was positioned on the cell surface with a three-dimensional hydraulic micromanipulator (MHW-3 Narishige Co., LTD). Current and voltage clamp recordings were performed using the perforated-patch recording technique (Rae et al., 1991) with use of Axopatch 200B patch clamp amplifier (Axon Instruments). Current signals were sampled at 5 KHz and were low-pass filtered at 1 KHz (six-pole Bessell). Digitized signals were further analyzed by personal computer using pClamp9.2 software (Axon Instruments). All experiments were performed at room temperature (20–25 °C).

2.4. Solution and chemicals

Amphotericin B (Sigma) stock solution was prepared by dissolving in dimethylsulfoxide (DMSO) (25 mg/ml). The pipette tip was initially filled with amphotericin B-free pipette solution by a brief immersion. The remainder of the pipette was then backfilled with the same solution also containing amphotericin B diluted to a final concentration of 240 μg/ml. Measurements were started after the stabilization of the membrane potential. The pipette solution contained (in mM) 140 KCl and 5 HEPES buffered to pH 7.4 (Tris). The bath solution contained (in mM): 140 NaCl, 10 KCl, 1.6 $CaCl_2$, 2 $MgCl_2$, 44 Glucose, and 2 HEPES buffered to pH 7.4 (Tris). The following drugs were used: tetrodotoxin (TTX) (Wako), scopolamine (Sigma), atropine (Sigma), mecamylamine (Sigma). Drugs were continuously perfused at flow rate of about 5 ml/min.

2.5. Statistical analysis

Values are given as the means ± SEM, with n representing the number of cells. For comparisons between two groups, Student's *t*-test, paired or unpaired was used for statistical analysis, with p values < 0.05 considered statistically significant.

3. Results

Amphotericin B perforated-patch recordings were used to record action potentials under the current-clamp condition, and to record net membrane currents under the voltage clamp condition in Kenyon cells freshly isolated from the mushroom body of the cricket *G. bimaculatus* brain.

3.1. Action potential and net membrane currents in Kenyon cells

Isolated Kenyon cells were generally rounded and the cell membrane capacitance measured in this study was 13.5 ± 0.8 pF (n = 59). Using a 140 mM KCl solution in the patch pipette, the average resting potential of Kenyon cells was −37 ± 2.4 mV (n = 15). The majority of cells used in this study did not exhibit spontaneous firing, but action potentials were elicited in response to depolarizing current injection. Fig. 1A_1 shows typical electrical responses elicited by depolarizing and hyperpolarizing current injections with the same stimulus intensity (10 pA). In this cell, the resting membrane potential was −47 mV. When an inward current of −10 pA was injected, the membrane potential hyperpolarized according to a single exponential function. When an outward current with the same stimulus intensity was injected, overshooting action potentials were elicited.

In order to understand the ionic mechanisms underlying the action potentials, net membrane currents in Kenyon cells were recorded under the voltage clamp condition. When depolarizing clamp pulses between −60 and +50 mV (10 mV increments, duration 300 ms) were applied from a holding potential of −60 mV, the inward currents followed by the outward currents were obtained (Fig. 1A_2). The inward currents were initially activated at −40 to −30 mV and peaked at −30 to −20 mV whereas the outward current initially activated at −30 to −20 mV and increased with membrane depolarization. The presence of two inward current components, Na^+ current (I_{Na}) and Ca^{2+} current (I_{Ca}) in Kenyon cells has been previously reported (Wüstenberg et al., 2004). Therefore, we tried to dissect the inward currents into sub-components by using ion substitution and selective blocking agents.

To block the I_{Ca}, we used a Ca^{2+}-free solution that was made equimolar by substituting $MgCl_2$ (1.6 mM) for $CaCl_2$. When the cell was perfused with this solution, both inward and outward currents were decreased by about 40 ± 6.2 (n = 12) and 33.2 ± 5.1% (n = 12), respectively (Fig. 1B_2). In Ca^{2+}-free solution, the inward currents were completely abolished by addition of 1 μM TTX to the bath solution (Fig. 1B_3). This result indicates that the inward current obtained in Ca^{2+}-free solution is I_{Na}. The difference currents obtained by

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