STRETCH-ACTIVATED ION CHANNELS IN CULTURED MECHANOSENSORY NEURONS OF *MANDUCA SEXTA*

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INTRODUCTION

Research into the properties of mechanoreceptor neurons lacks a cell culture model that would allow experimentation with fully differentiated neurons. Dissociation and culture of vertebrate mechanoreceptor neurons is not possible because their mechanosensitive endings are located far from their somata. Dissociated hair cells from cochlear and vestibular organs of several vertebrate species have been used to study their mechanical properties and their mechanically-activated whole-cell currents, but these preparations have not allowed reliable measurements of single-channel currents. Arthropod cuticular mechanoreceptors offer a better system for creating a culture of specialized mechanoreceptor neurons, because their sensory endings are often very close to the cell somata. We created a method for primary culture of mechanosensory neurons from pupal sphinx moth *Manduca sexta* antennae. This culture can be used for electrophysiological experiments in both the whole-cell and single-channel configurations.

PREPARATION

The antennae of adult *M. sexta* have both chemo- and mechanosensory functions. The distal part of each antenna is called the flagellum and it contains more than 250,000 olfactory neurons and several hundred mechanoreceptor neurons. The two basal segments, the scape and pedicel, have mainly mechanosensory functions. Both contain several fields of sensory hairs called Böhm's bristles and the pedicel contains the proprioceptive Johnston's organ. Because sensory neurons in adult animals are tightly wrapped with specialized glial and sheath cells, we used an early pupal stage for the cell dispersions. The sensory neurons appear during the second day after pupation and can be easily dissociated before they become wrapped by accessory cells.

CELL CULTURE

Enzymatic dissociation with 1mg/ml papain

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Cell disaggregation with DNAse + BSA

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Mechanical dissociation; trituration with siliconized Pasteur pipet

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Removal of debris; centrifugation with BSA

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Plating in HBSS

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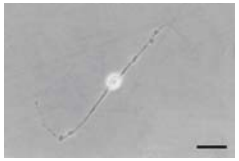
Growth in L-15 + conditioned Grace medium

BSA = Bovine Serum Albumin, Fraction V
HBSS = Hanks Balanced Salt Solution without calcium or magnesium
L-15 = Leibovitch L-15 medium
Conditioned Grace medium = Grace insect medium, collected from *M.sexta* embryonic cell line MRRL-CH1

ANTENNAL NEURONS IN CULTURE

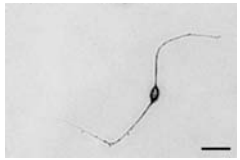
Cells dissociated from a whole antenna and grown 12 days in culture. Neurons (arrows) were easily distinguished from other cell types by their shiny somata and thin processes compared to the non-neural phase-dark cells. Scale bar 50 µm.



Cultures from the basal antenna had many large neurons, with soma diameters of 10-25 µm and usually two neurites that often deviated at their distal ends. Scale bar 50 µm.

ANTI-HORSERADISH PEROXIDASE (HRP) STAINING

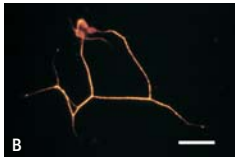
To reliably identify the neurons, we used anti-horseradish peroxidase staining, which is known to label neuron-specific molecules in insect neurons. The cultures were fixed with 4% paraformaldehyde, incubated in 1% H_2O_2, washed with 3% normal goat serum in PBT (=PBS + 0.1% triton X-100). Incubation with rabbit anti-peroxidase (1:1,000 in PBT) was performed for 3 hours at room temperature. After several rinses with 3% normal goat serum in PBT the cultures were incubated in the secondary antibody (1:500 peroxidase conjugated goat anti-rabbit IgG) for 2 hours. The staining was intensified by treatment with 0.3 mg/ml DAB.

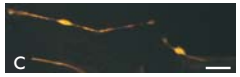


Anti-HRP antibody specifically stained bipolar neurons in all cultures. The spindle shaped large neurons with thick processes shown here were typical for cultures made from the basal antenna. Scale bars 50 µm.

MECHANOSENSORY PREFERRING ANTIBODY (MPA) STAINING

MPA is one of a series of monoclonal antibodies generated by Hishinuma et al. (1988) against *M. sexta* nervous tissue. MPA binds to neurofilaments that are typical for mechanosensory neurons, but not present in olfactory neurons. The cultures were fixed for 30 min in 4% paraformaldehyde, preincubated in milk powder buffer containing 3% rabbit serum, 0.1% triton X-100, 1% BSA and 3% skimmed milk powder in PBS, followed by an incubation in the primary antibody (1:100 in milk powder buffer). The next day the cultures were washed several times in milk powder buffer, and incubated in the secondary antibody (CY-3 1:600) for 1 hour.



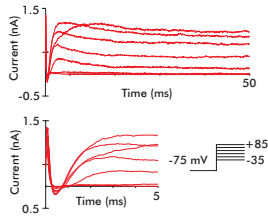


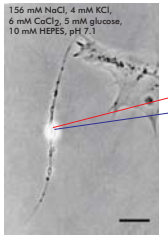
MPA staining was very specific. Staining was detected in the somata and varicosities of developing neurons during the first week in culture (A), but some neurites were stained more strongly already after 6 days in culture (B). The staining in the somata and neurites became more intense in older cultures (C 11 days and D 22 days in culture). The somata of large (diameter 10 - 25 µm) neurons was always stained, but in the small neurons (soma diameter 5 - 10 µm) staining was only detected in the neurites and in most of the neurons derived from the flagella MPA staining was not detected at all. Scale bars 50 µm.

ELECTROPHYSIOLOGY

WHOLE-CELL RECORDINGS

We performed whole-cell recordings from large bipolar neurons after 7-20 days in culture. All neurons had a large outward current that did not inactivate and a fast inward current superimposed on the outward current. These recordings were made without any blockers and we do not know if the inward current was carried by calcium or sodium ions. We did not find significant differences in current amplitudes at different ages. The maximum outward current amplitude was 815 pA (S.D. ±245 pA, n=7) and the inward current amplitude was 508 pA (S.D. ±331 pA. n = 7). Both currents activated at stimulus amplitudes of – -10 mV. The mean membrane resistance of these neurons was 1.5 GΩ (S.D. ±0.3 GΩ, n = 6).





PIPETTE SOLUTIONS

WHOLE-CELL

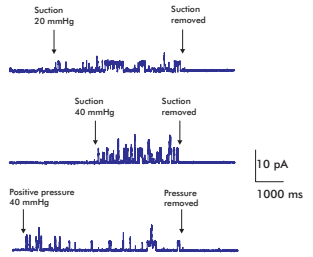
150 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$,
11 mM EGTA, 2 mM NaATP, 5 mM HEPES, pH 7.1

SINGLE-CHANNEL

140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$,
10 mM HEPES, pH 7.1

SINGLE-CHANNEL RECORDINGS

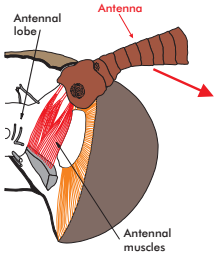
Single-channel recordings were made from cultures at 10-23 days. Cell-attached patches from somata always had one or more voltage-activated ion channel. We also found a high density of mechanosensitive ion channels on the somata. These channels responded equally strongly to both negative and positive pressures applied through the recording pipet. We also detected mechanosensitive ion channels on the neurites, but there were always many other active ion channels in these regions. The current amplitude of the most frequent mechanosensitive ion channel was ~3 pA. We also detected channels with current amplitudes of less than 1 pA at high voltages with similar stimulus strengths. The recordings shown here were made at a pipet potential of 50 mV, and this patch had 3 mechanosensitive ion channels with unitary conductances of 28 pS.

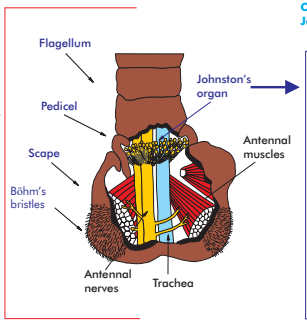


SUMMARY AND CONCLUSIONS

We have developed a primary cell culture system of antennal mechanoreceptor neurons from early stage pupal sphinx moth *Manduca sexta*. Dissociated neurons from the moth antenna differentiated, grew and survived for several weeks in a conditioned culture medium. Bipolar neurons with soma diameters of 10 - 25 µm from the basal portion of the antennae could be positively identified as mechanoreceptor neurons, presumably derived from the johnston's organ, using a monoclonal antibody that recognizes neurofilaments in these neurons. The immunoreactivity was clear and specific from the first day after dissociation and became stronger during several days in culture. These neurons appeared healthy and showed normal whole-cell currents only a few days after plating. We found numerous mechanosensitive ion channels responding to both negative and positive pressures on the somata and neurites of differentiated neurons. This new culture system provides access to mechanoreceptor neurons that has never been possible before, allowing the use of both mechanical and electrical stimuli on neurons that are free from the accessory structures surrounding them in intact preparations.

Manduca head





One mechanosensillum in Johnston's organ