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Acetylcholine receptors on cultured antennal cells of the moth *Manduca sexta*

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INTRODUCTION

Acetylcholine (ACh) is the most common neurotransmitter released from insect mechanosensory neurons to excite postsynaptic neurons, which mainly have nicotinic receptors. For example, the EPSPs in the motor neurons that are postsynaptic to sensory neurons in *Manduca sexta* (Trimmer and Weeks 1989) and locust (Leitch and Pitman 1995) can be blocked by α-bungarotoxin and other antagonists of nicotinic receptors. However, when multiple *Manduca* afferents were activated, or a train of stimuli in one afferent was elicited, an additional EPSP component could be recorded in the motor neurons. A similar response was elicited by oxotremorine, a specific agonist of muscarinic ACh receptors (mAChR) (Trimmer and Weeks 1989). Therefore, the motor neurons seemed to have both nicotinic and muscarinic ACh receptors. Surprisingly mAChR antagonists, such as scopolamine, increased the amplitude of EPSPs elicited by afferents in locust motor neurons (Judge and Leitch 1999), in cockroach giant interneurons that are postsynaptic to cercal afferents (Hue et al. 1989) and in larval *Manduca* motor neurons (Trimmer and Weeks 1989). These results suggest that there is a negative feedback loop from the postsynaptic neuron to the afferent via muscarinic autoreceptors located in the axon terminals of the afferents. Therefore, ACh also seems to mediate presynaptic inhibition in the mechanosensory afferents themselves. An alternative mechanism suggested by Judge and Leitch (1999) involves GABAergic interneurons having mAChRs that would be activated by ACh released from the afferents and then induce inhibition of the afferents themselves. However, little is known about the presence of ACh receptors in the peripheral sensory neurons of insects.

Antennal neurons of *Manduca* are mainly olfactory or mechanosensory and show choline acetyltransferase (ChAT) activity suggesting that they are cholinergic (Sanes and Hildebrand 1976). Acetylcholinesterase (AChE) has also been found in the mechanosensory neurons in the late pupal stage antenna (Stengl et al. 1990), but when cultured at an early pupal stage, these antennal neurons showed no AChE activity (Torkkeli and French 1999). Here, we studied the distribution of ACh receptors on cultured antennal neurons using fluorescent analogs of muscarinic and nicotinic receptor antagonists in order to learn whether these neurons are modulated by ACh.

METHODS

Adult sphinx moth *Manduca sexta* together with the pupal (*left*) and larval (*right*) stages.

Animals: A laboratory colony of the sphinx moth *Manduca sexta*

Preparation: Early stage pupae were placed on ice for 10 minutes. Antennae were dissected out, the antennal tissue was suspended in Hanks balanced salt solution (HBSS) and enzymatically dissociated with papain (1mg/ml). Cells were disaggregated with DNAse and further dissociated by mechanical trituration with a siliconized Pasteur pipet. Debris was removed by centrifugation with bovine serum albumin (BSA). The cells were resuspended in HBSS and plated on glass coverslips coated with a mixture of poly-L-lysine and concanavaline A. After attachment, a 2:1 mixture of Leibovitz's L-15 medium and conditioned Grace's medium was added. Cells were kept for up to a month in a 24°C humidified incubator and culture media was changed once a week

Staining: Fluorescent analogs (Molecular Probes) of the mAChR antagonist pirenzepine (Bodipy® FL) and the nAChR antagonist α-bungarotoxin (Alexafluor™) were used at concentrations of 50-200 nM and 1-5 µg/ml, respectively, in *Manduca* saline (156 mM NaCl, 4 mM KCl, 6 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES). After rinsing with *Manduca* saline, the cultured cells were incubated in the dye in the dark for one hour at room temperature. They were then rinsed twice with *Manduca* saline and observed with an inverted microscope under epifluorescent optics.

ELECTROPHYSIOLOGY

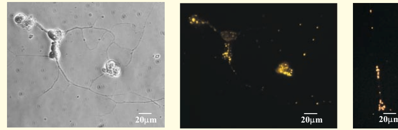
We used the whole-cell patch-clamp technique to test whether 1 mM ACh or 0.1 mM muscarine would elicit any currents in the cultured neurons. The pipet solution was (in mM): KCl 150; $MgCl_2$ 2; $CaCl_2$ 1; EGTA 11; Na^+ATP 2; HEPES 5; pH 7.1, adjusted to 330 mOsm. The bath solution consisted of (in mM): NaCl 156; KCl 4; $CaCl_2$ 6; Glucose 5; HEPES 10; pH 7.1, adjusted to 380 mOsm. The pipet resistance was 6 to 10 MΩ. The agonists were freshly dissolved in saline and applied directly to the bath solution. We could not see any clear responses to these agonists. Currents triggered by mAChR are usually small (peak current of 0.8 nA at a membrane potential of -50 mV in *Manduca* motoneurons), slow and can be inhibited by Ca^{2+}, Mg^{2+} or other divalent cations (Trimmer 1994).

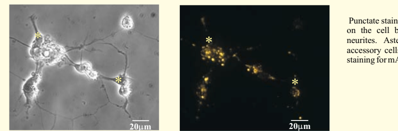
When both nicotinic and muscarinic receptors are present on the same cell, responses to ACh can be up to 40 times greater than to muscarine alone. In isolated neuronal somata of locusts, at a holding potential of -40 mV, the ACh current is about 12 nA, while a similar dose of muscarine elicits a current of approximately 300 pA (Benson 1992). Thus, the absence of large inward currents when the neurons are stimulated with ACh supports the idea that nicotinic AChR are not present on these cells.

The distribution of mAChR on antennal neurons depends on time in culture

Cultured antennal neurons shown under phase contrast optics (*left*) and the same neurons stained with the fluorescent analog of the mAChR antagonist pirenzepine under epifluorescent optics (*right)* at different times in culture.

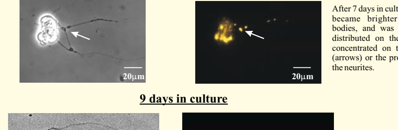
1 day in culture





Punctate staining can be seen on the cell bodies and the neurites. Asterisks indicate accessory cells that show no staining for mAChR.

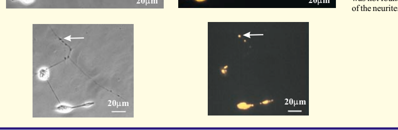
7 days in culture



After 7 days in culture the staining became brighter on the cell bodies, and was not as widely distributed on the neurites, but concentrated on the varicosities (arrows) or the proximal parts of the neurites.

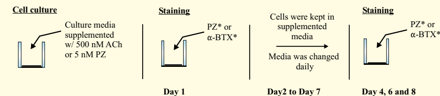
9 days in culture

17 days in culture



After 17 days in culture the staining was very bright on the cell bodies and the varicosities (arrows), but it was not found on other parts of the neurites.

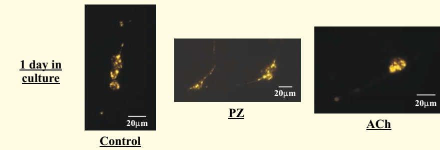
Co-incubation with ACh downregulates the expression of mAChR



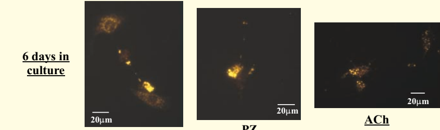
ACh: Acetylcholine ; **PZ**: Pirenzepine ; **PZ***: Labeled pirenzepine ; α-BTX*: Labeled α-bungarotoxin

Cultured antennal neurons labeled for mAChR and shown under epifluorescent optics at different times in culture. In neurons supplemented with ACh the staining was weak after 6 days in culture and completely absent or very faint after 8 days in culture, while in controls and neurons supplemented with unlabeled pirenzepine the staining remained strong.

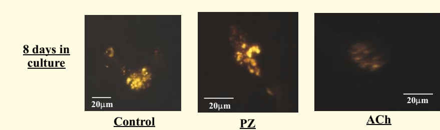
1 day in culture



6 days in culture



8 days in culture



SUMMARY AND CONCLUSIONS

Fluorescent analogs of muscarinic and nicotinic receptor antagonists were used to assess the distribution of ACh receptors on cultured antennal neurons of the moth *Manduca. sexta* at different stages of development. The effects of chronic treatments with muscarinic agonist and antagonist on the distribution of mAChR were also investigated.

1. Nicotinic ACh receptors were not seen in any of the cells in these mixed cultures.
2. Muscarinic ACh receptors were found in all neurons as well as in some of the accessory cells. The distribution of the mAChR varied with time in culture: Punctate staining was initially found in all parts of the neurons, including the neurites. After several days or weeks in culture the staining was restricted to the cell bodies and varicosities.
3. Co-incubation with unlabeled pirenzepine, did not induce any visible change in the staining pattern. However, staining in the control cultures was very intense, making it difficult to observe possible changes by eye.
4. Co-incubation with ACh significantly reduced the number of receptor binding sites, suggesting a down-regulation of the mAChR expression. This effect may be caused by increased receptor degradation and/or decrease in the synthesis of receptor protein because of the decreased levels of its mRNA.

Taken together, these findings indicate that the sensory neurons in *Manduca* antenna have a high density of mACh receptors and that they cluster to strategic locations during development. While the location of receptors in cultured neurons may not be exactly the same as in intact antennae, the receptors were especially accumulated on the somata, and this may mean that they are targets for ACh coming from sources other than the neurons' own endings that are located far from the somata. Therefore, our results argue against the autoreceptor theory (Trimmer and Weeks 1989). There is no evidence for efferent innervation of the peripheral parts of *Manduca* mechanoreceptors or chemoreceptors and therefore it is possible that ACh is synthesized and released in non-neural cells. Future research in our laboratory will be aimed at discovering the source of this transmitter.

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