

Voltage- and Calcium-activated Currents in Cultured Olfactory Receptor Neurons of Male *Mamestra brassicae* (Lepidoptera)

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Abstract

Insect olfactory receptor neurons (ORNs) grown in primary cultures were studied using the patch-clamp technique in both conventional and amphotericin B perforated whole-cell configurations under voltage-clamp conditions. After 10–24 days *in vitro*, ORNs had a mean resting potential of -62 mV and an average input resistance of 3.2 G Ω . Five different voltage-dependent ionic currents were isolated: one Na⁺, one Ca²⁺ and three K⁺ currents. The Na⁺ current (35–300 pA) activated between -50 and -30 mV and was sensitive to 1 μ M tetrodotoxin (TTX). The sustained Ca²⁺ current activated between -30 and -20 mV, reached a maximum amplitude at 0 mV (-4.5 ± 6.0 pA) that increased when Ba²⁺ was added to the bath and was blocked by 1 mM Co²⁺. Total outward currents were composed of three K⁺ currents: a Ca²⁺-activated K⁺ current activated between -40 and -30 mV and reached a maximum amplitude at $+40$ mV (605 ± 351 pA); a delayed-rectifier K⁺ current activated between -30 and -10 mV, had a mean amplitude of 111 ± 67 pA at $+60$ mV and was inhibited by 20 mM tetraethylammonium (TEA); and, finally, more than half of ORNs exhibited an A-like current strongly dependent on the holding potential and inhibited by 5 mM 4-aminopyridine (4-AP). Pheromone stimulation evoked inward current as measured by single channel recordings.

Introduction

For their survival, insects depend heavily on their capacity to monitor their external chemical environment. They have evolved very sensitive and specific olfactory receptor neurons (ORNs). This sensitivity and specificity of insect ORNs, in addition to the availability of active ligands, make insects useful models for the study of olfaction.

Insect ORNs are bipolar neurons located on the antennae. The dendrites extend into cuticular structures—sensilla—and the axons project to central nervous structures—the antennal lobes—where olfactory information is processed. These sensillar structures are particularly suitable for recording odorant responses extracellularly (Kaissling, 1986). An abundant literature describes the high specialization of insect ORNs, the detection of sex pheromone compounds by male moths being the prime example. However, in contrast to vertebrate main olfactory epithelium or vomeronasal organ, where sensory neurons can be either acutely isolated (Maue and Dionne, 1987a; Firestein and Werblin, 1989; Liman and Corey, 1996) or patch-clamp recorded from intact epithelial preparation (Ma *et al.*, 1999; Leinders-Zufall *et al.*, 2000), the presence of the cuticle impedes direct access to the ORNs in insects. There are thus very few

descriptions of currents or ion channels expressed in insect ORNs.

To elucidate the processes underlying olfactory transduction, it is necessary to have an understanding of the electrophysiological properties of ORNs. The activation of voltage-dependent ion channels, involved in the generation of action potentials, constitutes an important step in ORN response. They play important roles in shaping spike firing patterns and are likely involved in the high sensitivity of ORNs. Adult insect ORNs could only be isolated from *Locusta migratoria* (Wegener *et al.*, 1992). High conductance Ca²⁺- and voltage-dependent K⁺ channels, ATP blockable, present on the soma of these neurons, were characterized in single channel recordings. In Lepidoptera, ORNs were isolated from nymphs and before cuticle deposit (Zufall *et al.*, 1991a) at an early stage of their development. These neurons expressed only one voltage-gated current, a transient outward current with the characteristics of an A-current. *In situ* patch-clamp recordings from insect ORNs, either on extruded dendrite (Zufall and Hatt, 1991) or cell body (Dubin and Harris, 1997), are feasible but technically difficult due to the small size of these sensory cells and the presence of closely enveloping accessory cells and the

cuticle. Moreover, *in situ* recordings limit the use of pharmacological agents. The development of long-term ORN cultures (Stengl and Hildebrand, 1990) has helped to resolve many of these difficulties. Patch-clamp recordings demonstrated the expression of four different types of voltage-gated channels in cultured ORNs from *Manduca sexta*: one Na⁺ and three K⁺ channels (Zufall *et al.*, 1991b). The sustained outward current had the characteristics of the delayed rectifier. The transient outward current presented the properties of the A-current. Single Ca²⁺-activated K⁺ channels were described in inside-out recordings, but no Ca²⁺-activated K⁺ current was characterized in whole-cell recordings.

Biochemical (Boekhoff *et al.*, 1990a,b; Breer *et al.*, 1990) and electrophysiological data (Wegener *et al.*, 1993; Stengl, 1994) demonstrate the involvement of IP₃ as the second messenger of olfactory transduction in insects. An IP₃-dependent Ca²⁺ current, a Ca²⁺-dependent cation current and a protein kinase C-dependent cation current are successively activated in pheromone receptor neurons after pheromone stimulation (Stengl, 1994). These three inward currents may constitute the receptor potential that elicits a discharge of action potentials encoding odor quantity and quality centrally. However, in apparent contradiction with these observations, olfactory dendrites of *Antheraea polyphemus* seem to be equipped with only one type of ion channel, that is sex-pheromone-dependent (Zufall and Hatt, 1991). These ion channels were activated from the intracellular side by protein kinase C activators such as diacylglycerol and phorbol ester and by cGMP, but not by Ca²⁺ or IP₃. This discrepancy indicates that insect olfactory transduction is still not fully elucidated.

We have developed a procedure to culture olfactory neurons (Lucas and Nagnan-Le Meillour, 1997). These cell cultures provide a useful system for the study of sensory signaling and its modulation in insects. As a first step, we present in this paper new data concerning voltage-gated currents that are expressed in insect ORNs. We found that cultured insect ORNs possess a variety of voltage- and Ca²⁺-dependent currents, including an Na⁺ current, a Ca²⁺ current, a Ca²⁺-dependent K⁺ current, a delayed-rectifier K⁺ current and a rapidly inactivating K⁺ current (A-current). Although they support previous findings, several new results, including the characterization of a voltage-gated Ca²⁺ current and a macroscopic Ca²⁺-activated K⁺ current, are presented here for the first time in insect ORNs. Moreover, we have begun to study the responsiveness of insect ORNs *in vitro*. These results have already been presented in abstract form (Lucas and Shimahara, 2001).

Materials and methods

All culture media were purchased from Life Technologies In Vitro Gene and all chemicals from Sigma, except

tetrodotoxin (TTX) which was purchased from Alomone Laboratories.

Insects

Mamestra brassicae (Lepidoptera, Noctuidae) were reared on a semi-artificial medium (Poitout and Bues, 1974). Larvae were maintained at 20–24°C under a long photoperiod regimen (16 h light/8 h dark) and 40–50% humidity. Experimental pupae were selected within 12 h after pupation and were kept at 23°C until dissected to isolate their antennal flagella.

Primary cultures of antennal neurons

The cell culture protocol was slightly modified from the method previously described (Lucas and Nagnan-Le Meillour, 1997) in order to improve the survival of antennal cells. All steps of this protocol were carried out at room temperature. Briefly, antennal flagella from 4-day-old male pupae were dissected in 3 + 2 medium (three parts of Leibovitz's L15 medium and two parts of Grace's medium supplemented with lactalbumin hydrolysate and yeastolate). The antennal tissue was placed in 1 mg/ml EGTA for 5 min and rinsed three times for 5–10 min in Hanks' Ca²⁺- and Mg²⁺-free salt solution (HBSS). Then, flagella were disrupted by 20 min of incubation in L-cystein-activated papain (1 mg/ml), rinsed three times in HBSS and carefully triturated with a fire-polished Pasteur pipette. The resulting cell suspension was plated onto uncoated 35 mm Falcon Petri dishes. Dissociated cells were allowed to settle and to adhere to the surface of culture dishes for at least 30 min. The culture medium was then replaced with 150 µl of a 2 + 1 mixture of L15 medium conditioned with 5% of fetal calf serum and Grace medium supplemented with lactalbumin hydrolysate and yeastolate and conditioned on the embryonic cell line MRRL-CH1 (Eide *et al.*, 1975; Stengl and Hildebrand, 1990). The cultures were then inverted to form a hanging column and were maintained at 23°C in a humid atmosphere. Half of the culture medium was changed every 4–7 days.

Typically, cells were found to survive *in vitro* for >6 weeks. Coating of the dish surface was not necessary since non-neuronal cells, probably glial cells (Lucas and Nagnan-Le Meillour, 1997), multiplied and formed a continuous sheet to which other cell types attached (Figure 1). ORN-like cells that were previously found to exhibit positive anti-HRP staining (Lucas and Nagnan-Le Meillour, 1997) were distinguished from other cell types on the basis of their small cell bodies (5–8 µm) and their thin processes.

Recording solutions

During recordings, cells were kept in ~1 ml of 'extracellular' Ringer solution containing (in mM): NaCl, 156; KCl, 4; CaCl₂, 6; glucose, 5; and HEPES, 10, adjusted to pH = 7.4 with NaOH. In some recordings, Ca²⁺ was replaced with Ba²⁺ (Ca²⁺-free Ringer) or Na⁺ was replaced by choline

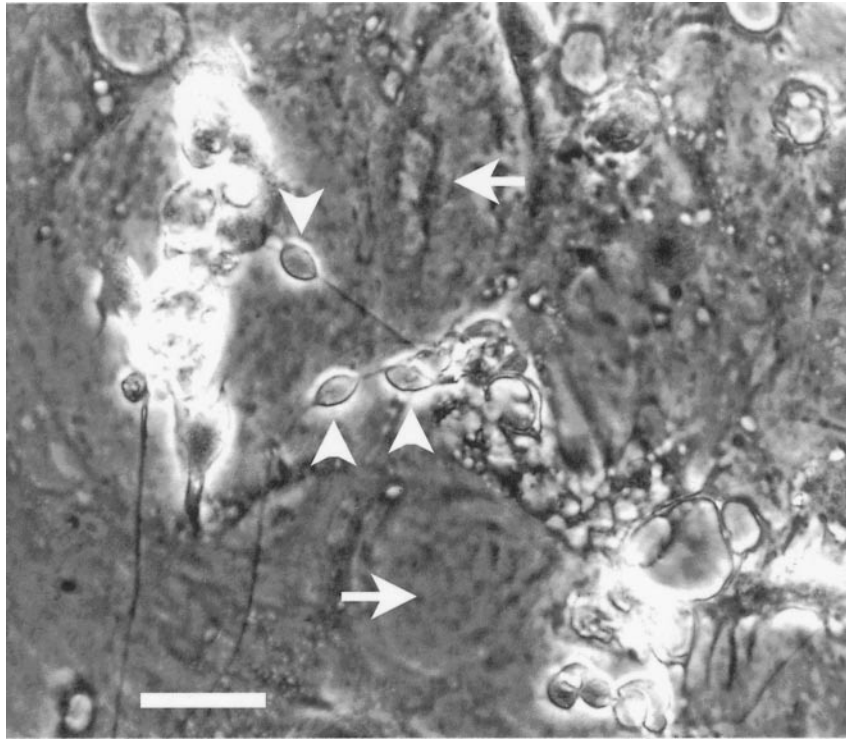


Figure 1 View of a 14-day-old culture of antennal cells of *M. brassicae*. Within 5 days after plating dissociated antennal cells, non-neuronal cells (arrows), most probably glial cells, covered the surface of the dish and formed a continuous sheet to which other cells attached. Three ORN-like cells (arrowheads) grew and extended their processes on this cell layer. Scale bar = 10 μm .

(Na^+ -free Ringer). To increase the Ca^{2+} current amplitude, in some recordings Ba^{2+} (10, 20 or 40 mM) was added to Ca^{2+} . NaCl concentration was reduced according to the concentration of BaCl_2 to maintain the osmotic pressure of the solution. Recordings were performed for a maximum of 2 h after the replacement of the culture medium with the extracellular recording solution. The 'intracellular' pipette filling solution used for whole-cell recordings contained (in mM): KCl , 150; NaCl , 5; MgCl_2 , 2; CaCl_2 , 1; HEPES , 10; EGTA , 11; adjusted to pH 7.2 with KOH . The free Ca^{2+} concentration in the pipette solution was calculated to be 18 nM. In some experiments, all K^+ in the pipette solution was replaced with Cs^+ and 20 mM tetraethylammonium (TEA) in order to block outward currents. Perforated patch-clamp recording electrodes were filled with (in mM): Cs-MeSO_4 , 130; CsCl , 25; CaCl_2 , 1; HEPES , 10; EGTA , 11; adjusted to pH 7.2 with CsOH . The osmolarities of bath and intracellular solutions were adjusted when necessary to 360 and 330 mosmol/l, respectively, by the addition of mannitol. The compositions of the different solutions used are summarized in Table 1.

Drug and pheromone application

A gravity driven system was used to perfuse the cells at a flow rate of 1.5 ml/min. Different solutions could be perfused using a mechanical rotary valve. For pheromone stimulation, a pipette was filled with 10 μM (Z)-11-hexa-

decenyl acetate (Z11-16:Ac; synthesized in the laboratory by M. Lettere). Z11-16:Ac is the main pheromone component of *M. brassicae* (Bestmann *et al.*, 1978; Descoins *et al.*, 1978). It activates one of the two neurons housed in each long sensillum trichodeum present on male antennae (Den Otter and Van der Hagen, 1989; Renou and Lucas, 1994). Z11-16:Ac was dissolved in dimethyl sulfoxide (DMSO), with the final concentration of DMSO in Ringer not exceeding 0.1%. DMSO alone did not elicit any current on ORNs. Recombinant pheromone binding protein Mbra-BBP1 (Campanacci *et al.*, 1999), which specifically binds *in vitro* Z11-16:Ac (Maïbeche-Coisne *et al.*, 1997), was added at a concentration of 50 μM to help solubilize hydrophobic pheromone and better activate pheromone receptor proteins. Pheromone stimuli were pressure ejected towards the cell body of the neuron being recorded using a picospritzer (General Valve company, Fairfield, NJ). Fluorescein was used in a set of preliminary experiments to establish the optimal placement of the stimulus pipette (50 μm from the cell), its tip diameter (2 μm) and the ejection pressure (2×10^4 Pa). By measuring fluorescence dilution after injection of a defined concentration of fluorescein in these conditions, the mean volume ejected in 1 s was measured to be ~ 10 nl. The deduced amount of pheromone ejected in the direction of the recorded neuron was 10^{-14} mol. During pheromone application, the membrane patch was kept at 0 mV in the cell-attached configuration.

Table 1 Composition of solutions

	Ringer	Ringer Na ⁺ -free	Ringer Ba ²⁺ 10	Ringer Ba ²⁺ 20	Ringer Ba ²⁺ 40	Ringer Ca ²⁺ -free	Pipette	Pipette Cs ⁺ TEA ⁺	Pipette perforated
NaCl	156		141	126	96	156	5		
CholineCl		158							
KCl	4	2	4	4	4	4	150		
CaCl ₂	6	6	6	6	6		1	1	1
EGTA							11	11	11
Glucose	5	5	5	5	5	5			
Hepes	10	10	10	10	10	10	10	10	10
MgCl ₂							2	2	
BaCl ₂			10	20	40	6			
CsCl								130	25
CsMeSO ₄									130
TEACl								20	
pH	7.4 (NaOH)	7.4 (KOH)	7.4 (NaOH)	7.4 (NaOH)	7.4 (NaOH)	7.2 (NaOH)	7.2 (KOH)	7.2 (CsOH)	7.2 (CsOH)

All concentrations are given in mmol/l.

Patch-clamp technique and data analysis

All recordings were obtained at room temperature from ORNs that had been in culture for 10–24 days. Neurons were identified on the basis of their morphology at 600× magnification with an Olympus IX 70 inverted microscope equipped with phase-contrast and Hoffmann modulation contrast optics. Currents were recorded using conventional patch-clamp recording methods (Hamill *et al.*, 1981), with an Axopatch 200 B amplifier (Axon Instruments, Union City, CA). Electrodes were pulled from thick-wall borosilicate glass capillaries (World Precision Instruments, GC150-10) using a programmable micropipette puller (model P97; Sutter Instruments, Novato, CA). The pipettes were coated with Sylgard (GE Bayer Silicones, The Netherlands) and fire polished. When filled with the physiological solutions, their tip resistance was 4–6 MΩ for whole-cell or perforated recordings and 7–10 MΩ for cell-attached recordings. The recording dish was grounded using an agar bridge to an Ag/AgCl electrode. Pipette offset potential was compensated before forming a seal. The cell was approached with positive pressure in the pipette and the high resistance seal (>10 GΩ and frequently >40 GΩ) was either formed spontaneously when removing the pressure or when gentle suction was applied. Before breaking into the whole-cell configuration, the pipette capacitance was compensated and a potential of –60 mV was applied in the electrode, preventing the cell from experiencing a loss in its holding potential. The whole-cell configuration was obtained by applying further suction in the pipette.

For some recordings, the perforated patch-clamp technique with amphotericin B was employed to prevent run-down of Ca²⁺ currents according to the protocol described previously (Rae *et al.*, 1991). A stock solution of amphotericin B was prepared daily by dissolving 3 mg of amphotericin B in 50 µl

DMSO. The recording pipette was filled by dipping its tip into the intracellular saline solution for ~10 s. The pipette was then back-filled with a solution containing 4 µl of the stock solution of amphotericin B vigorously stirred for 1 min with 1 ml of intracellular pipette solution. The amphotericin B dilution was used for a maximum of 2 h. The access resistance to the cell interior was monitored under pCLAMP 8 by applying 10 mV pulses. In most cases, it reached a stable value between 15 and 40 MΩ within 1–10 min.

Data were acquired and analyzed with the aid of pCLAMP 8 software (Axon Instruments, Union City, CA). Cells were clamped at a holding potential of –60 or –80 mV and voltage steps were used to activate voltage-gated channels in the neurons. For whole-cell recordings, a fractional (P/N) method, using four fractionally scaled hyperpolarized sub-pulses, was used for online leak compensation. Resting membrane potentials were measured as zero-current potentials. Cell capacitance was calculated by membrane test algorithms of pCLAMP 8 as the integration of the area under the capacitive current evoked by 10 mV voltage pulses from a holding potential of –60 mV. Membrane resistance was either determined from the slope of the current–voltage (*I–V*) relationship between –150 and –50 mV, where no voltage-gated currents were elicited, or it was read from membrane test protocols of pCLAMP 8 as solved iteratively using the Newton–Raphson method. Currents were low-pass filtered at 2–5 kHz with a low-pass four-pole Bessel filter and digitally sampled at 20 kHz. In whole-cell recordings, series resistance (33.5 ± 13.0 MΩ, *n* = 48) was compensated (80%) when the whole-cell current was >300 pA. On average, the remaining 20% of uncompensated series resistance will have caused an error (underestimation) in command potential of ~6.7 mV for

each nanoampere of whole-cell current. The amplitude of sustained currents was measured as the mean amplitude of the current elicited during the second half of 100 or 200 ms voltage steps. The amplitude of transient currents was measured as the maximum of the current elicited within 5 ms (Na^+ current) or 25 ms (A-like current) after voltage steps.

Mean values \pm standard deviations (SD) are given throughout and n indicates the number of neurons.

Results

Whole-cell passive properties

The duration of recordings was between 5 and 30 min. Whole-cell capacitance ranged from 2.0 to 5.5 pF (3.6 ± 1.0 pF, $n = 27$). The resting potential as measured immediately after breaking into the whole-cell mode varied from -48 to -75 mV (-62 ± 7 mV, $n = 42$). The membrane resistance of the cells ranged from 1.3 to 10.3 G Ω (3.2 ± 2.2 G Ω , $n = 25$). No correlation was found between age of neurons in culture (10–24 days) and their membrane capacitance, resting potential or input resistance.

Inward currents

Na^+ current

A fast transient inward current activated between -50 and -30 mV and reached a maximal amplitude that ranged from 35 to 300 pA at -10 or 0 mV (Figure 2a). The amplitude of the transient inward current was probably underestimated, since it declined rapidly with time and because most cells were not well space clamped, as indicated by the delay before recording inward current in response to steps to -40 mV (Figure 2a). Despite efforts to lower the access resistance to the cells, we were unable to improve the space clamp. ORNs in culture were probably not electrically compact due to their long and thin processes. The transient inward current was inhibited by the addition of TTX (10^{-6} M). It was not observed when Na^+ was replaced with choline in the bath ($n = 3$). Thus, this current presented the characteristics of an Na^+ current. In some recordings, trains of action potentials were observed (Figure 2c). This incomplete voltage clamp of the site of generation of action potentials indicates that Na^+ channels were probably not located on the soma, but rather on neurites.

Ca^{2+} current

In the search for voltage-dependent Ca^{2+} current, outward currents were blocked by replacing K^+ with Cs^+ and by adding 20 mM TEA into the patch pipette; Na^+ current was inhibited by the addition of 1 μM TTX into the bath. Due to its extremely small amplitude and rapid run down, the sustained inward current was recorded using the perforated patch-clamp technique with amphotericin B. Currents elicited by steps were repeated three times for each potential and averaged to improve the signal-to-noise ratio. Under

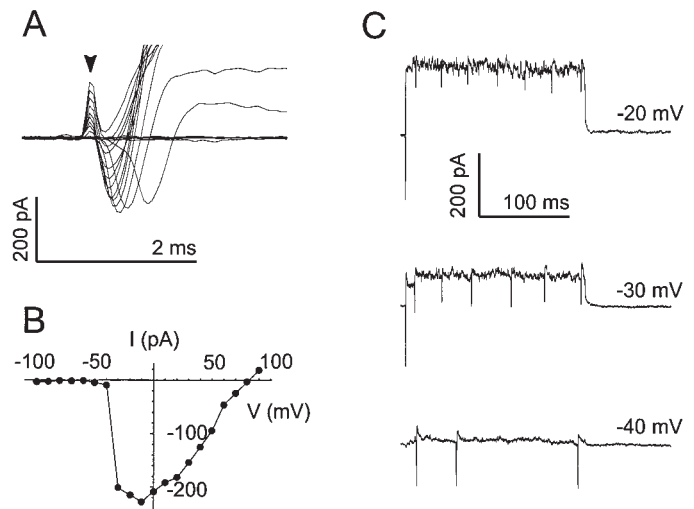


Figure 2 Time-course of Na^+ current in response to depolarizing voltage steps from a holding potential of -60 mV. Normal Ringer and pipette solutions were used. (A) Transient inward currents at a high time resolution. A capacitive artefact (arrowhead) preceded the Na^+ current. (B) Current-voltage relation of Na^+ current. Data are from the recording shown in (A). (C) Trains of action potentials elicited by depolarizing pulses from a holding potential of -60 mV.

these conditions, a sustained inward current was elicited by depolarizing voltage steps (Figure 3a). It activated between -30 and -20 mV and reached a maximum amplitude (-4.5 ± 6.0 pA, $n = 18$) at 0 mV. The amplitude of this inward current was correlated to the concentration of Ba^{2+} present in the bath solution (Figure 3b): -9.7 ± 8.4 pA ($n = 7$) at 0 mV in 10 mM Ba^{2+} ; -14.4 ± 10.1 pA ($n = 7$) at 10 mV in 20 mM Ba^{2+} ; and -26.5 ± 12.2 pA ($n = 9$) at 10 mV in 40 mM Ba^{2+} . With Ba^{2+} in the bath solution, this inward current was observed in all recorded ORNs ($n = 23$) and it presented some inactivation during 100 ms voltage steps (Figure 3c). This inward current, recorded with 40 mM Ba^{2+} , was blocked when 1 mM Co^{2+} was added to the bath ($n = 5$; Figure 3d). Taken together, these results indicate that all ORNs expressed a sustained inward current carried by Ca^{2+} . Its little inactivation when recorded with Ba^{2+} in the bath solution could indicate that more than one type of Ca^{2+} channel underlies the Ca^{2+} current.

In order to check for the presence of a low threshold T-type current, 100 ms depolarizing voltage steps were applied from a holding potential of -80 mV ($n = 6$). In no case was a rapidly decaying inward current evoked at potentials more negative than -40 mV, indicating that no T-type channel was expressed in cultured ORNs.

Outward currents

In standard Ringer, all ORNs from which recordings were made exhibited a sustained voltage-activated outward current and $\sim 10\%$ of ORNs ($n = 12$) exhibited a transient outward current after depolarizing voltage steps. When K^+ was replaced with Cs^+ + TEA in the recording pipette,

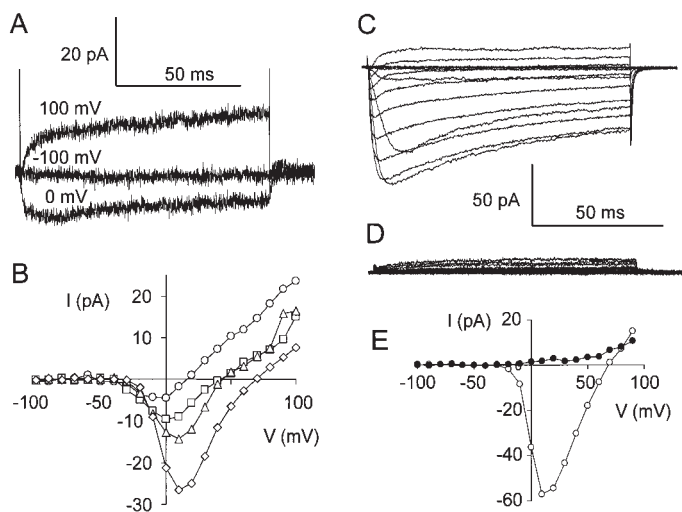


Figure 3 Perforated patch-clamp recordings of Ca^{2+} current. Na^+ and K^+ currents were blocked, respectively, by the addition of 1 mM TTX to the bath and by replacing K^+ with Cs^+ and adding 20 mM TEA in the electrode. **(A)** Whole-cell current elicited by voltage steps of 100 ms duration from a holding potential of -60 mV in normal Ringer. For clarity, only steps to -100 , 0 and $+100$ mV are presented. A sustained inward current was elicited in response to depolarizing steps. Its amplitude was maximal at 0 mV. **(B)** Mean current–voltage curves of current elicited by voltage steps of 100 ms duration in normal bath solution ($n = 18$, circles), in bath with 10 mM Ba^{2+} ($n = 7$, squares), in bath with 20 mM Ba^{2+} ($n = 8$, triangles) and in bath with 40 mM Ba^{2+} ($n = 8$, diamonds). **(C)** Whole-cell current elicited by voltage steps of 100 ms duration in 10 mV increments from a holding potential of -60 mV in bath with 40 mM Ba^{2+} . An inward current presenting some inactivation was elicited. **(D)** The inward current was inhibited within a minute after the addition of 1 mM Co^{2+} to the bath. Same cell as in **(C)**. **(E)** Current–voltage curves from data shown in **(C)** (open circles) and **(D)** (filled circles).

the outward current was almost completely inhibited (11.9 ± 8.6 pA at $+60$ mV, $n = 18$), indicating that the outward current was carried by K^+ ions. The analysis of outward current revealed three different K^+ currents: a Ca^{2+} -activated K^+ current, a delayed-rectifier K^+ current and an A-like current.

Ca^{2+} -activated K^+ current

The sustained voltage-dependent outward current activated at -40 mV and exhibited no or a weak decrement during the 100 or 200 ms voltage steps, indicating a slow inactivation (Figure 4a). In every recorded ORN it showed an N-shaped I – V curve (Figure 4c), as typically observed when a Ca^{2+} -gated K^+ current is activated. The current peaked between 30 and 40 mV, with an amplitude ranging from 202 to 2068 pA (734 ± 483 pA, $n = 30$). The I – V curve became a simple rising function within a minute after the addition to the bath of two Ca^{2+} channel inhibitors: 1 mM Co^{2+} ($n = 7$; Figure 4b, c) or 10 μM Cd^{2+} ($n = 2$). Co^{2+} had no effect at 100 μM ($n = 2$). In standard Ringer, the N shape of the I – V curve faded spontaneously within a few minutes with a similar time course to Ca^{2+} current rundown and progressively became a simple rising function (Figure 4e). The Ca^{2+} -

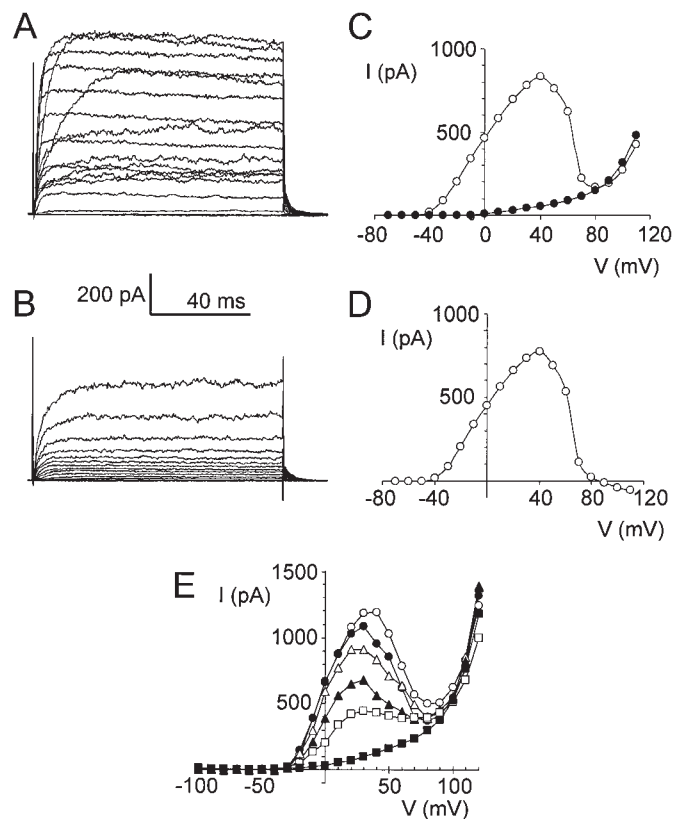


Figure 4 Ca^{2+} -dependent K^+ current. **(A)** Outward current elicited by voltage steps of 100-ms duration from -70 to $+110$ mV in 10 mV increments from a holding potential of -60 mV. The Na^+ current was blocked by the addition of 1 mM TTX to the bath. This cell expressed only a sustained outward current. Normal Ringer and pipette solutions were used. **(B)** Same cell as in **(A)** after the addition of 1 mM Co^{2+} to the bath. Only a slowly activating, non-inactivating outward current was activated. **(C)** Current–voltage relation of the outward current shown in **(A)** (open circles) and **(B)** (filled circles). **(D)** Current–voltage relation of the subtracted Co^{2+} -sensitive current (Ca^{2+} -dependent K^+ current) from recordings shown in **(A)** and **(B)**. **(E)** Current–voltage relations of sustained current elicited by voltage steps from -100 to $+120$ mV in 10 mV increments from a holding potential of -60 mV. Responses to voltage steps were recorded at 90 s (open circles), 150 s (filled circles), 225 s (open triangles), 360 s (filled triangles), 540 s (open squares) and 630 s (filled squares) after breaking into the whole-cell configuration. The last recording was made 75 s after addition of 1 mM Co^{2+} .

dependent component, calculated as the subtracted Co^{2+} -sensitive outward current, showed a bell-shaped I – V curve (Figure 4d), whose maximal amplitude, at $+40$ mV, was 605 ± 351 pA ($n = 7$). This sustained current was classified as a Ca^{2+} -dependent K^+ current. The potential at which the Ca^{2+} -dependent K^+ current was maximum was 40 mV higher than the potential that elicited the maximum of Ca^{2+} current in normal Ringer. Therefore, the Ca^{2+} -dependent K^+ current was also dependent on the membrane potential.

Two pharmacological agents were used to determine if intracellular Ca^{2+} stores were involved in the generation of Ca^{2+} -dependent K^+ current, as observed in vertebrate ORNs (Zufall *et al.*, 2000). Thapsigargin, a potent inhibitor of

Ca^{2+} -ATPases that mediate the uptake of cytosolic Ca^{2+} into endoplasmic reticulum stores, and ryanodine, an inhibitor of Ca^{2+} release from sarcoplasmic reticulum, were applied in the bath. Neither 1 μM thapsigargin ($n = 4$) nor 20 μM ryanodine ($n = 6$) were found significantly to modify the magnitude of the sustained outward current. Therefore, the Ca^{2+} -dependent K^+ current did not depend on the release of intracellular Ca^{2+} .

Delayed-rectifier K^+ current

In normal Ringer, depolarizations beyond potentials that activate Ca^{2+} -dependent K^+ current (≥ 80 mV) activated a sustained current with a slower kinetics than weaker depolarizations (Figure 5a). This slowly activating Ca^{2+} -independent K^+ current was isolated after the addition of 1 mM Co^{2+} . It activated between -30 and -10 mV (Figure 5b) and had an I - V relation that followed single exponential curves (Figure 5c). The mean amplitude of the current elicited by a step to $+60$ mV from a holding potential of

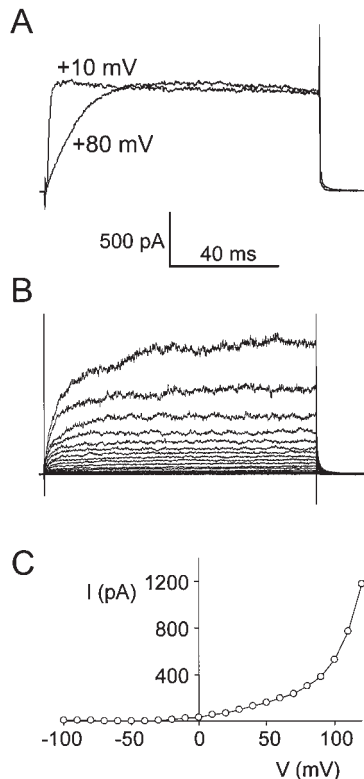


Figure 5 Delayed-rectifier K^+ current. **(A)** Current elicited by steps to $+10$ and $+80$ mV from a holding potential of -60 mV. In response to these two steps, the current reached a similar steady state level but it activated faster at $+10$ mV, where the Ca^{2+} -dependent K^+ current is largely predominant, than at $+80$ mV, where the sustained outward current is mainly composed of the delayed-rectifier K^+ current. Normal Ringer and pipette solutions were used. **(B)** Outward current recorded in response to voltage steps from -100 to $+120$ mV in 10 mV increments from a holding potential of -60 mV after the addition of 1 mM Co^{2+} to Ringer. Normal pipette solution was used. **(C)** Current-voltage relation of the Co^{2+} -independent K^+ current shown in (B). This current has the characteristics of the delayed-rectifier K^+ current.

-60 mV at least 2 min after addition of 1 mM Co^{2+} in the bath was 111 ± 67 pA ($n = 7$). In Ca^{2+} -free Ringer, a similar current had an amplitude of 124 ± 65 pA ($n = 12$). The Ca^{2+} -independent sustained outward current presented the characteristics of the delayed-rectifier K^+ current. This current had no run down, was not inhibited by the addition of 5 mM 4-aminopyridine (4-AP; $n = 3$) and was strongly reduced by the addition of 20 mM TEA ($n = 5$).

Transient K^+ current (A-like current)

In standard Ringer, $\sim 10\%$ of neurons ($n = 12$) exhibited a transient outward current at the beginning of depolarizing voltage steps (Figure 6a). This transient outward current typically inactivated in < 40 ms and was followed by a sustained K^+ current. The ratio between peak and sustained currents varied considerably between individual ORNs. In more than half of the ORNs where it appeared to be absent, a transient outward current was unmasked by equimolar replacement of all external Ca^{2+} ions with Ba^{2+} ions (9 out of 15 ORNs; Figure 7b) or after the addition of 10 μM Cd^{2+} in the bath (one out of two ORNs). The transient outward current was completely inactivated when the holding potential was set to values equal or more positive than -40 mV (Figure 7d) and it was inhibited by the addition of 5 mM 4-AP to the bath ($n = 3$; Figure 7e). Thus, the transient outward current presented similarities with the A-current

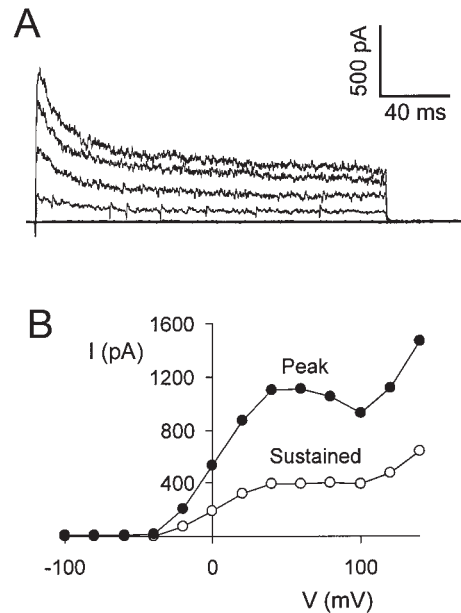


Figure 6 Time course of outward current. **(A)** Current elicited by voltage steps of 200 ms duration from -100 mV to $+40$ mV in 20 mV increments from a holding potential of -60 mV. Currents to steps from 60 to 140 mV are not presented for clarity. This cell expressed a rapidly activating and inactivating current that dominated during the first 50 ms of steps and a sustained current that was visible during the second half of steps. Normal Ringer and pipette solutions were used. **(B)** Current-voltage relation of the transient (filled circles) and sustained (open circles) outward currents shown in (A).

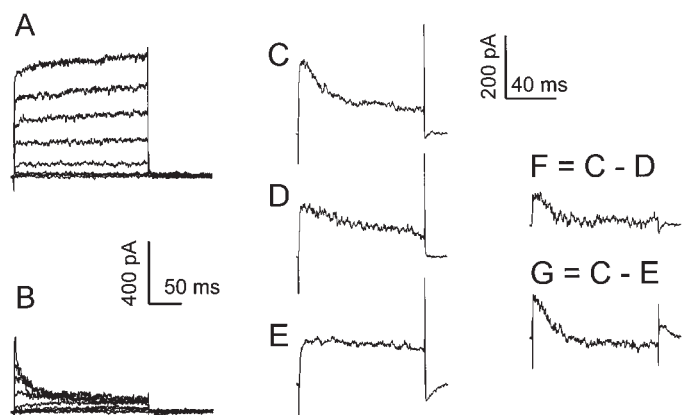


Figure 7 Transient K^+ current. **(A)** Whole-cell current elicited by voltage steps of 100 ms duration from -100 to $+60$ mV in 20 mV increments from a holding potential of -60 mV. **(B)** Same cell as in **(A)** in Ca^{2+} -free Ringer. A transient outward current inactivated in <40 ms and was followed by a sustained outward current. **(C)** Current elicited by a step to $+60$ mV from a holding potential of -80 mV in Ca^{2+} -free Ringer. The Ca^{2+} -dependent K^+ current was not activated and the remaining current had two components: a transient K^+ current that inactivated in ~ 40 ms and a sustained current. (**C**–**G** were recorded from a different cell to **A** and **B**). **(D)** Current elicited by a step to $+60$ mV from a holding potential of -40 mV in Ca^{2+} -free Ringer. Only a sustained outward current with some slow inactivation was elicited. **(E)** Current elicited by a step to $+60$ mV from a holding potential of -80 mV in Ca^{2+} -free Ringer containing 5 mM 4 -AP. The transient component of the outward current was blocked by 4 -AP. **(F)** Subtracted current of (**C**)–(**D**) shows the transient outward current inactivated at -40 mV. **(G)** Subtracted current of (**C**)–(**E**) shows the transient outward current blocked by 5 mM 4 -AP.

first described in gastropod neurons (Connor and Stevens, 1971). In contrast to Cd^{2+} treatment or replacement of extracellular Ca^{2+} with Ba^{2+} , the A-like current was never unmasked by the addition of Co^{2+} to the bath. On the contrary, when visible in standard extracellular solution, the A-like current was inhibited by 1 mM Co^{2+} application ($n = 2$).

Hyperpolarization-activated current

No inward current was activated by 200 ms hyperpolarization up to -150 mV. However, since inward rectifying K^+ current (I_h) activates slowly in frog ORNs (Trotier and Døving, 1996), we also tested the effects of longer hyperpolarizing pulses. Still, no current was activated during 5 s hyperpolarizing pulses from -70 to -110 mV in 10 mV increments ($n = 5$). The mean current measured during the second half of the 5 s pulses to -110 mV was -2.3 ± 2.7 pA.

Responses to olfactory stimulation

Cultured neurons were tested for responses to pheromone stimulation. A mixture of the major component of the female sex pheromone, Z11-16:Ac, and the PBP that specifically binds this compound *in vitro*, MbraPBP1, was used as pheromone stimulus. In the cell-attached configuration at 0 mV holding potential, no ion channel openings

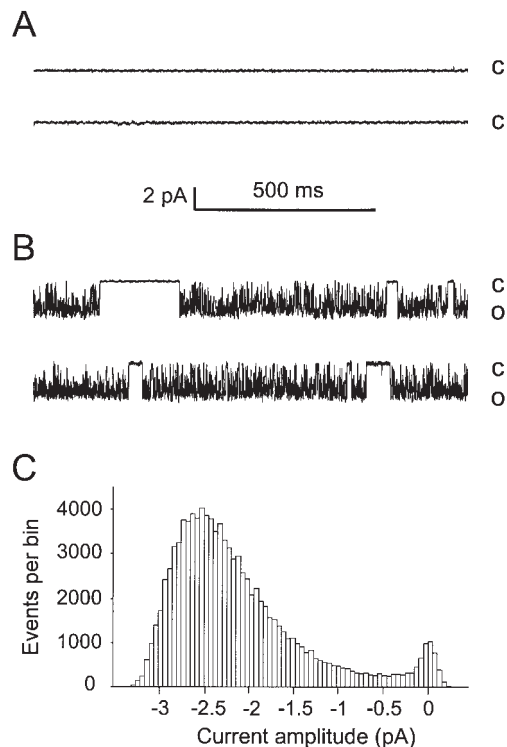


Figure 8 Pheromone-dependent currents in cultured ORNs. Normal Ringer and pipette solutions were used. **(A)** At the resting potential (0 mV in the recording electrode) in the cell-attached configuration no ion channel openings were recorded. **(B)** After stimulating the ORN with a mixture of Z11-16:Ac (10 μ M) and Mbra-PBP1 (50 μ M), ion channel openings were recorded. In the absence of washing, the ion channel opening activity remained constant for 16 min without any sign of adaptation. **(C)** Amplitude histogram of pheromone-activated ion channel openings indicates that probably only one type of ion channel was activated by pheromone stimulation. Data are from the recording shown in **(A)**.

were recorded from the soma before stimulation (Figure 8a). After pheromone stimulation, inwardly directed ion channel openings with rapid flickering activity were recorded in 3 out of 14 cells (Figure 8b). In the absence of bath exchange, this ion channel activity continued after the end of pheromone application. The amplitude histogram of channel openings indicated that only one channel was activated by pheromone stimulation (Figure 8c). In the absence of pheromone application, such channel openings had never been observed in the cell attached configuration ($n > 100$).

Discussion

Our observations indicate that ORNs isolated from 4 -day-old pupae and grown in culture for at least 10 days expressed at least five different types of voltage-gated currents. Furthermore, these cultured neurons could respond to the main component of the pheromone by opening pheromone-gated ion channels. These are indications of preserved physiological functions *in vitro*.

The measured resting potential of *M. brassicae* ORNs, -62 ± 7 mV, was remarkably similar to the values measured

from other insect ORNs: -62 mV from *M. sexta* cultured ORNs (Zufall *et al.*, 1991b) and -70 mV *in situ* from *Drosophila* ORNs (Dubin and Harris, 1997). Current clamped ORNs usually displayed spontaneous fluctuations in membrane potentials that probably resulted from spontaneous channel activity. As already observed in ORNs, a large measured input resistance (3.2 ± 2.2 G Ω) implies that very few ion channels were open at rest. This confers a very high sensitivity to these neurons, as in vertebrate ORNs where action potential firing could be elicited by injections of only 3 pA (Firestein and Werblin, 1987).

A transient inward current was blocked by TTX and was not observed in Na^+ -free Ringer. It was classified as an Na^+ current (I_{Na}). The morphology of *M. brassicae* ORNs *in vitro*, with long (several hundreds of micrometers) and thin processes prevented good space clamping of these neurons, similarly to *in situ* whole-cell recordings of *Drosophila* ORNs (Dubin and Harris, 1997). Therefore, I_{Na} amplitude (between 35 and 300 pA) was probably underestimated. Action potentials recorded from some neurons under voltage-clamp whole-cell conditions indicate that the site of generation of action potentials is not located on the cell body. This is consistent with the findings that Na^+ channels were never observed on the soma of *M. sexta* cultured ORNs (Zufall *et al.*, 1991b) or isolated mouse ORNs (Maue and Dionne, 1987b). Furthermore, in isolated lobster ORNs, the amplitude of Na^+ current depended on the presence of a remnant axon and not on the size of the soma (McClintock and Ache, 1989).

A sustained TTX-resistant inward current was blocked by Co^{2+} . This current could also be carried by Ba^{2+} and exhibited a rapid run-down. It was classified as Ca^{2+} current (I_{Ca}). This current activated between -40 and -30 mV and had a maximum amplitude at 0 mV that was shifted to $+10$ mV when 20 or 40 mM Ba^{2+} was added to the bath. The activation threshold of I_{Ca} was closer to that of high-voltage-activated (HVA) Ca^{2+} currents (~ -30 mV) than to that of low-voltage-activated (LVA) Ca^{2+} currents (~ -60 mV) (Wicher *et al.*, 2001). Moreover, it presented a slow inactivation during 100 ms steps. Thus, I_{Ca} presented characteristics close to HVA Ca^{2+} currents. No LVA (T-type) Ca^{2+} current was discovered. A similar small voltage-gated Ca^{2+} current, with peak Ca^{2+} current <10 pA, has been described in fish (Corotto *et al.*, 1996). This is much smaller than in toad ORNs, where Ca^{2+} currents were reported to be >200 pA (Delgado and Labarca, 1993). Despite its small amplitude under physiological conditions (-4.5 ± 6 pA at 0 mV), I_{Ca} plays an important role in *M. brassicae* ORNs by activating a large Ca^{2+} -dependent K^+ current. Voltage-gated Ca^{2+} currents have been characterized in a number of vertebrate ORNs, as well as in squid and lobster ORNs (Schild and Restrepo, 1998), but had never been described in insects. The Ca^{2+} current may have been overlooked due to its small amplitude in insects. In recordings from *M. sexta* ORNs (Zufall *et al.*, 1991b), voltage-gated Ca^{2+} channels

may have been inhibited by concanavalin A, used to coat the substrate on which ORNs were cultured (Stengl and Hildebrand, 1990). Such an inhibition has been demonstrated in a ciliate (Ivens and Deitmer, 1986).

Outward currents were much larger than inward currents and were entirely blocked by loading the cell with Cs^+ , indicating that they were carried by K^+ . We have identified three types of K^+ current on the basis of their kinetics and pharmacology: (i) a Ca^{2+} -dependent K^+ current ($I_{\text{K(Ca)}}$); (ii) a delayed-rectifier K^+ current ($I_{\text{K,DR}}$); and (iii) a rapidly inactivating K^+ current (I_{A}). The relative amplitudes of these three K^+ currents varied considerably from cell to cell, but the main component of the sustained outward current was always $I_{\text{K,Ca}}$. The association of these three different K^+ currents or ion channels in ORNs appears to be conserved from vertebrates [rodents (Maue and Dionne, 1987b; Lynch and Barry, 1991a,b), fish (Miyamoto *et al.*, 1992; Corotto *et al.*, 1996) and amphibians (Firestein and Werblin, 1987; Schild, 1989)] to invertebrates [squid (Lucero and Chen, 1997) and insects (Zufall *et al.*, 1991b)].

Our observation of $I_{\text{K(Ca)}}$ in *M. brassicae* is the only characterization of the macroscopic Ca^{2+} -dependent K^+ current in insect ORNs. This current was absent in Ca^{2+} -free Ringer and was completely inhibited by 1 mM Co^{2+} . By contrast, in *M. sexta* cultured ORNs, $I_{\text{K(Ca)}}$ is not blocked by Cs^+ or any other conventional blockers (Stengl, 1993), suggesting differences between moth species. In *M. brassicae*, $I_{\text{K(Ca)}}$ activated very fast, suggesting that Ca^{2+} -dependent K^+ channels are colocalized with Ca^{2+} channels. The absence of effect of thapsigargin and ryanodine on outward current amplitude indicates that $I_{\text{K(Ca)}}$ did not depend on intracellular Ca^{2+} stores. $I_{\text{K(Ca)}}$ was dependent both on potential and Ca^{2+} , as indicated by the shift to the right by $+40$ mV of $I-V$ curves of $I_{\text{K(Ca)}}$ compared to those of I_{Ca} . Potassium channels that may account for $I_{\text{K(Ca)}}$ have been described in invertebrate ORNs. In *Locusta migratoria* ORNs, a K^+ channel located on soma and dependent both on membrane potential and intracellular Ca^{2+} concentration, was classified as a maxi- K^+ channel due to its high conductance (180 pS) and strong K^+ selectivity (Wegener *et al.*, 1992). This channel was half-activated for a free Ca^{2+} concentration of 10^{-7} M. A similar Ca^{2+} and voltage-dependent K^+ channel, with a mean conductance of 215 pS, was identified in lobster ORNs (McClintock and Ache, 1989). By contrast, in *M. sexta* a Ca^{2+} -activated K^+ channel with a smaller conductance, 66 pS, showed little voltage dependence (Zufall *et al.*, 1991b). In mouse ORNs, two different Ca^{2+} -activated K^+ channels have been characterized, one voltage-dependent and the other voltage-insensitive (Maue and Dionne, 1987b). While the macroscopic current activated in *M. brassicae* ORNs demonstrates the presence of voltage-dependent Ca^{2+} -gated K^+ channels, it does not rule out the expression of their voltage-insensitive counterparts. The expression of a large and fast activating $I_{\text{K(Ca)}}$ in *M. brassicae* ORNs suggests that this current contributes to

the fast repolarization that allows insect ORNs to respond to rapid stimulations (Rumbo and Klaissling, 1989; Marion-Poll and Tobin, 1992). The sensillum lymph that bath outer dendritic segments *in situ* has an unusually high (200 mM) K^+ concentration (Klaissling and Thorson, 1980). Thus, to be involved in repolarization, K^+ channels must be located in membranes exposed to low external K^+ levels, such as the inner dendritic segment, the soma or the axon. In bursting neurons, Ca^{2+} -activated K^+ channels have also been implicated in the control of interspike interval and firing frequency (Hille, 1992).

A slowly activating K^+ current was isolated in all neurons by blocking $I_{K(Ca)}$ either with 1 mM Co^{2+} or the replacement of external Ca^{2+} with Ba^{2+} . Both procedures selectively blocked $I_{K(Ca)}$ without much effect on the remaining sustained outward current that had the characteristics of a delayed-rectifier K^+ current, $I_{K,DR}$. It activated between -30 and -10 mV. At $+60$ mV, the amplitude of $I_{K,DR}$ averaged 111 ± 67 pA after addition of 1 mM Co^{2+} and 124 ± 65 pA in Ca^{2+} -free Ringer. Delayed-rectifier K^+ channels were characterized from invertebrate ORNs. These 30 pS channels were the most common in cell-attached recordings from the soma of cultured *M. sexta* ORNs (Zufall *et al.*, 1991b). In lobster ORNs, delayed-rectifier K^+ channels had a lower conductance of 9.7 pS (McClintock and Ache, 1989).

A transient outward current was clearly visible in $\sim 10\%$ of ORNs when recorded in standard external Ringer. The same transient current, with inactivation kinetics, voltage dependence and 4-AP sensitivity similar to an A-current (Hille, 1992; Wicher *et al.*, 2001) was unmasked in more than half of ORNs after inhibition of $I_{K(Ca)}$ in Ca^{2+} -free Ringer. The relative amplitude of I_A to the complete outward current was highly variable. This current was blocked by 1 mM Co^{2+} . However, I_A was not dependent on Ca^{2+} concentration, since it could be observed in Ca^{2+} -free Ringer. Therefore, the effect of Co^{2+} was most likely direct on transient K^+ channels. I_A is the first voltage-dependent current expressed in immature insect ORNs (Zufall *et al.*, 1991a). However, it remains unclear if all immature ORNs express this current. It was isolated from 50% of ORNs in larval salamanders (Firestein and Werblin, 1987) and was not found in toad (Delgado and Labarca, 1993) or lobster ORNs (McClintock and Ache, 1989). Our sampling was too small to study a correlation between I_A amplitude and the age of ORNs. Developmental studies are needed to determine if two different types of neurons exist or if I_A amplitude decreases to zero during neuron lifetime. I_A currents are classically described as being involved in shaping action potentials and in modulating spike frequencies (Connor and Stevens, 1971; Hille, 1992). It may also contribute, in cooperation with $I_{K(Ca)}$, to the repolarization of action potentials, provided that the I_A channels are located *in situ* in the inner dendritic segment, the soma or the axon.

A Cl^- channel, independent from the membrane potential,

was characterized in lobster ORNs (McClintock and Ache, 1989). In vertebrate ORNs, the cAMP-mediated olfactory transduction cascade involves entry of Ca^{2+} through cyclic-nucleotide-gated channels and leads to the opening of Ca^{2+} -gated chloride channels (Kleene and Gesteland, 1991; Kleene, 1993; Lowe and Gold, 1993). Opening of these channels depolarizes the cell, since the reversal potential for Cl^- is more positive than the resting potential. In our recording conditions, when internal K^+ was replaced by Cs^+ and TEA, a small sustained outward current remained. This outward current could be carried either by K^+ , due to incomplete dialysis of the Cs^+ pipette solution with the cytosol, or by Cs^+ if its permeability through K^+ channels is different from zero, as has been already demonstrated in squid ORNs (Lucero and Chen, 1997). A third possibility would be that this outward current was carried by Cl^- . However, because of its small amplitude (11.9 ± 8.6 pA at $+60$ mV, $n = 18$), we did not attempt to block this current with Cl^- channel blockers. Regardless, under the conditions tested, *M. brassicae* ORNs did not display a significant Cl^- conductance.

In contrast to vertebrate (Lynch and Barry, 1991c; Miyamoto *et al.*, 1992; Trotier *et al.*, 1993; Trotier and Døving, 1996) and lobster ORNs (Corotto and Michel, 1994), neither an inwardly rectifying K^+ current, nor a hyperpolarization-activated cation conductance were observed in *M. brassicae* ORNs. A cAMP- and hyperpolarization-activated ion channel, with a weak selectivity for K^+ over Na^+ , has been cloned from a cDNA library of *Heliothis virescens* (Krieger *et al.*, 1999). *In situ* hybridization revealed a localization of this channel in cell bodies beneath sensilla, possibly ORNs. Our experiments showed no indication of such a channel in *M. brassicae* ORNs. If it is present, this channel must be expressed at too low a density to elicit any macroscopic current. By contrast, it is tempting to speculate that this ion channel is, rather, expressed in accessory cells. This would be consistent with our findings of hyperpolarization-activated conductances in accessory cells (Lucas, 1999).

We have begun to study the responsiveness of cultured ORNs to pheromones. The major component of the pheromone of *M. brassicae*, Z11-16:Ac, elicited a response in 3 out of 14 ORNs. Non-responding neurons were perhaps sensitive to minor pheromone components or to heterospecific compounds that inhibit male attraction to pheromone sources. *In situ*, these compounds activate ORNs insensitive to Z11-16:Ac (Renou and Lucas, 1994). Responses consisted of the opening of inwardly directed ion channel openings after pheromone application. This is in good agreement with previous recordings of olfactory responses from *M. sexta* cultured ORNs (Stengl *et al.*, 1992). In the absence of bath exchange, channel activation by pheromone application lasted for several minutes and did not show any sign of adaptation. Channels opened in long bursts and presented a flickering activity similar to

pheromone-dependent cationic channels recorded from the dendritic membranes of ORNs (Zufall and Hatt, 1991) or from the soma of cultured ORNs (Stengl, 1993). It is conceivable that ion channels located in dendritic membranes of ORNs *in situ* might be expressed on the soma of cultured neurons. This has been demonstrated in cultured lobster ORNs, where odors evoked currents in ORNs without processes, suggesting that these neurons could *in vitro* insert all the elements of the transduction cascade into their soma, including those normally confined to processes (Fadool *et al.*, 1993). Our *in vitro* preparation will allow the study of the role and specificity of PBPs in pheromone activation.

This work extends the number of invertebrate species, that included one single moth species, in which voltage-gated currents were characterized in ORNs. This *in vitro* preparation and these results will give the basis for future studies on olfactory transduction, as well as for dissecting the mechanisms of action of olfactory modulators or blockers of pheromone responses in insects.

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