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Ca^{2+} Stabilizes the Membrane Potential of Moth Olfactory Receptor Neurons at Rest and Is Essential for Their Fast Repolarization

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Abstract

The role of Ca^{2+} in insect olfactory transduction was studied in the moth *Spodoptera littoralis*. Single sensillum recordings were made to investigate in vivo the role of sensillar Ca^{2+} on the electrophysiological properties of sex pheromone responsive olfactory receptor neurons (ORNs). Lowering the sensillar Ca^{2+} concentration to 2×10^{-8} M increased ORN spontaneous firing activity and induced long bursts of action potentials (APs) superimposed on spontaneous negative deflections of the transepithelial potential. We inferred that Ca^{2+} stabilizes the membrane potential of ORNs, keeping the spontaneous firing activity at a low and regular level. Neither the amplitude and kinetics of the rising phase of sensillar potentials (SPs) recorded in response to pheromone stimuli nor the AP generation during stimulation depended on the extracellular Ca^{2+} concentration. Thus, extracellular Ca^{2+} is not absolutely necessary for ORN response. Partial inhibition of responses with a calmodulin antagonist, W-7, also indicates that intracellular Ca^{2+} contributes to the ORN response and suggests that Ca^{2+} release from internal stores is involved. In 2×10^{-8} M Ca^{2+}, the repolarization of the SP was delayed when compared with higher Ca^{2+} concentrations. Therefore, in contrast to depolarization, ORN repolarization depends on extracellular Ca^{2+}. Ca^{2+}-gated K^+ channels identified from cultured ORNs with whole-cell recordings are good candidates to mediate ORN repolarization.

Key words: calcium signaling, depolarization, insect olfactory transduction, patch-clamp, repolarization, single sensillum recording

Introduction

In insects, the detection of odors eliciting attraction or avoidance behaviors is often a question of life or death and is thus essential for species survival. Insects have evolved highly sensitive olfactory systems, the highest specialization being observed in moth pheromone communication where males can detect minute quantities of the female sex pheromone blend (Kaissling and Priesner 1970). As a consequence, pheromone detection in moths has become the best-studied model of insect olfaction (Kaissling 2004; Jacquin-Joly and Lucas 2005).

Insect olfactory receptor neurons (ORNs) are bipolar cells housed in sensilla located on the antennal flagella. The binding of pheromone molecules to specific receptors present on the outer dendrites of ORNs activates a chemo-electrical transduction cascade that converts the odorant–receptor interaction into a graded electrical response. This cascade is a multistep process that includes the production of second messengers, the opening of second messenger–gated channels leading to the receptor potential, and finally the activation of voltage-dependent channels triggering action potentials (APs) (Stengl et al. 1999; Jacquin-Joly and Lucas 2005). Trains of APs then encode information about the quality, intensity, and temporal pattern of the stimuli (Kaissling 1986). Next, this olfactory information is conveyed to the antennal lobes where it is further processed (Hansson and Anton 2000).

The molecular mechanisms of olfactory transduction are being deciphered using biochemical, electrophysiological, and molecular genetic techniques. In insects, pheromone reception involves the activation of phospholipase C (PLC) (Boekhoff, Raming, and Breer 1990; Boekhoff, Strotmann, et al. 1990; Boekhoff et al. 1993), leading to the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Patch-clamp experiments performed on insect ORNs grown in primary cultures indicated that both IP_3 (Stengl 1994) and DAG (Lucas and Pézier 2006) open Ca^{2+}-permeable channels and thus can be considered as second messengers. Stengl (1993, 1994) proposed that the transient rise in intracellular Ca^{2+} opens Ca^{2+}-dependent channels that amplify the depolarization, leading to the generation of the receptor potential. Such a 2-step olfactory process, with a Ca^{2+} entry through second messenger–gated channels followed by a Ca^{2+}-mediated amplification of the depolarization, has been described in vertebrate ORNs (Schild and Restrepo 1998) and likely also occurs in vertebrate vomeronasal sensory neurons (VSNs) (Bigiani et al. 2005; Jacquin-Joly and Lucas 2005). Second messengers, cAMP in ORNs (Nakamura and Gold 1987) and DAG in VSNs (Lucas et al. 2003), activate Ca^{2+}-permeable cationic channels. The resulting rise in intracellular Ca^{2+} concentration activates an excitatory conductance (Kleene 2002; Liman 2003). In addition, Ca^{2+} modulates the odor transduction pathway at various stages (Menini 1999; Matthews and Reisert 2003).

The aim of the present work is to study in vivo the role of Ca^{2+} in insect pheromone transduction and more specifically to address 4 questions. 1) Does Ca^{2+} modulate the firing activity of ORNs at rest? 2) Is Ca^{2+} involved in insect ORN depolarization in vivo, as previously postulated on the basis of in vitro experiments? 3) Does Ca^{2+} release from intracellular stores play a role in the transduction cascade as demonstrated in vertebrate ORNs (Zufall et al. 2000) and suggested in insect ORNs (Stengl 1993)? 4) Is Ca^{2+} implicated in the termination of the insect olfactory response, as is the case in vertebrates (Reisert and Matthews 1998; Dougherty et al. 2005)?

To address these questions, we used the single sensillum recording (SSR) technique. This technique allows us to manipulate the composition of the sensillum lymph in which outer dendritic segments of ORNs are bathed. We can, by a passive perfusion, add pharmacological agents as well as proteins to the sensillar lymph through the open tip of sensilla from the recording electrode (Redkozubov 2000a; Pophof 2002; Pophof and Van Der Goes Van Naters 2002). We monitored under low, intermediate, and high extracellular Ca^{2+} concentrations the following electrical events: 1) The transepithelial potential (TEP) that is the potential difference recorded between the sensillum lymph and the hemolymph. The TEP is generally considered to be the standing potential produced by the electrogenic activity of the accessory cells (Thurm and Wessel 1979). 2) The sensillar potential (SP), a slow negative deflection of the TEP evoked by odor stimulation. The SP reflects the relative variation of the dendritic membrane potential as a function of stimulus intensity (Vermeulen and Rospars 2001); we analyzed SPs to estimate the dependence of the receptor potential on extracellular Ca^{2+} concentration. 3) The firing of APs in absence of olfactory stimulation (spontaneous firing activity) or following a puff of pheromone (firing response).

Our in vivo experiments confirmed the involvement of extra- and intracellular Ca^{2+} in insect olfactory transduction. In particular, we demonstrated that extracellular Ca^{2+} plays a crucial role by stabilizing the resting membrane potential of unstimulated ORNs and is essential for the quick repolarization of the ORNs after response to odors.

Materials and methods

Insects

Spodoptera littoralis was reared on an artificial diet at 20 °C or 25 °C under a long-day photoperiod (16:8 h light:dark) (Poitout et al. 1972). Pupae were sexed, and males and females were kept separately. Three-day-old male pupae were selected for primary cell cultures and were kept at 20 °C. One- to 3-day-old adult males were used for SSRs.

Single sensillum recordings

Two physiological types of *sensilla trichodea* have been described in *S. littoralis* males. Sensilla from the most numerous type contain at least one neuron highly tuned to (Z,E)-9,11-tetradecadienyl acetate (Z9,E11-14:Ac), the main pheromone component; these sensilla are distributed over the ventral antennal surface. Sensilla of the other type are restricted to the lateral edges of antennal segments and contain 2 ORNs responding to 2 other compounds (Ljungberg et al. 1993; Quero et al. 1996). In the present study, SSRs with the tip-recording method (Kaissling and Thorson 1980) were performed from whole male-insect preparations on long *sensilla trichodea* responding to Z9,E11-14:Ac and located on the 8th–15th proximal segments from the base of the antenna. The recording electrode, a glass electrode with a tip diameter of about 7 μm, was slipped over the cut end of one hair. To minimize contributions of field potentials, the reference electrode was inserted into an adjacent segment.

We used the tip-recording technique to assess the role of sensillar Ca^{2+} on the electrophysiological properties of ORNs. This method requires cutting the tip of the sensillum that can result in cutting the dendritic tip of ORNs. In the tip-recording method, ORNs had a low and stable spontaneous firing activity and were able to respond to odorant stimuli for long periods of time after sensilla were cut (>6 h), indicating that the ORNs were in good physiological state. In particular, dendritic tip excision most probably did not induce any significant increase in intracellular Ca^{2+} concentration because intracellular perfusions of cultured ORNs with a Ca^{2+} concentration above 1 μM rapidly activate depolarizing currents leading to AP generation (Stengl 1993). It is thus reasonable to assume that the dendritic membrane rapidly reseals after the dendrite tip has been cut, preventing a physiologically important rise in intracellular Ca^{2+} concentration. Moreover, Ca^{2+} extrusion through exchangers must occur, as in vertebrate ORNs (Schulze et al. 2002), so that Ca^{2+} concentration rapidly returns to a basal level.

To our knowledge, no pharmacological agent can block selectively all the Ca^{2+}-permeable ion channels potentially located on the outer dendrite. To modify Ca^{2+} entry into ORNs, we thus modified the extracellular sensillar Ca^{2+} concentration using different electrode solutions. Kaissling and Thorson (1980) designed 2 Ringer solutions based on the analysis of the ionic composition of the sensillum lymph and the hemolymph of *Antherea polyphemus*. These solutions are widely used in tip recordings from diverse moth species to fill, respectively, the reference electrode, which contacts the hemolymph, and the recording electrode, which contacts the sensillar lymph. The sensillar lymph Ringer has a 10^{-3} M Ca^{2+} concentration and supports long-term recordings of the activity of insect ORNs. Thus, 10^{-3} M Ca^{2+} was taken as the control condition for the saline filling the recording electrode, and it was compared with salines having a higher (6×10^{-3} M) or a lower (2×10^{-8} M) Ca^{2+} concentration (Table 1). The low Ca^{2+} concentration was chosen on the basis of whole-cell patch-clamp recordings on cultured ORNs of *S. littoralis* (Pézier and Lucas 2006) and *Manduca sexta* (Stengl 1993) because no Ca^{2+}-dependent currents are activated when the intracellular Ca^{2+} concentration is maintained at 2×10^{-8} M. We thus took 2×10^{-8} M as an estimate of the Ca^{2+} concentration in the outer dendrite of ORNs at rest. This concentration is close to the resting Ca^{2+} concentration inside olfactory cilia of salamander ORNs, which was estimated at ca. 4×10^{-8} M (Leinders-Zufall et al. 1998). We thus lowered the extracellular Ca^{2+} concentration to 2×10^{-8} M to reduce the Ca^{2+} entry through Ca^{2+}-permeable ion channels in an attempt to diminish the intracellular activation of Ca^{2+}-dependent channels. The 2×10^{-8} M free Ca^{2+} concentration was obtained using 4 mM ethyleneglycol-bis(2-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetra-acetic acid (EGTA) with 0.02 mM Ca^{2+} as calculated with WebmaxC v.2.20 (Table 1).

Recordings were started less than a minute after connecting the recording electrode to a sensillum and lasted 35 min. In some experiments, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7; Sigma, Saint Quentin Fallavier, France) was added in the recording electrode solution. W-7 is a membrane permeable calmodulin antagonist that has been widely used in insect neurons (Courjaret and Lapied 2001; Seno et al. 2005). W-7 stock solution (100 mM) was prepared in dimethyl sulfoxide (Sigma). Final dilution of W-7 (100 μM) contained 0.1% dimethyl sulfoxide. This concentration of solvent was found to have no effect on electrophysiological properties of ORNs.

A humidified and charcoal-filtered airflow (70 l/h) was continuously directed at the preparation. Pheromone stimulations were obtained by blowing a puff of air (200 ms, 10 l/h) through a Pasteur pipette containing 500 ng of Z9,E11-14:Ac (M. Lettere, INRA). The small diameter of the tip of the Pasteur pipette (1.2 mm) and its short distance from the recording site (3 mm) allowed a localized stimulation of about 5 segments. Pheromone stimulations were applied every 10 min starting 1 min after covering the sensillum tip with the recording electrode.

The biologic signal was recorded on 2 channels using a Neurolog NL 102 amplifier. It was amplified (×100) and filtered (DC to 5000 Hz) to record SPs on one channel. On a second channel, it was amplified (×1000) and filtered (150–5000 Hz) to record only APs. A thermistor placed upstream of the stimulation cartridge allowed us to monitor the stimulation on a third channel. The three signals were sampled at 10 kHz through a 12-bit acquisition card (DT3001, Data Translation, Marlboro, MA) driven by Awave software (Marion-Poll 1995) and stored on a PC.

SPs were analyzed with Clampfit 9.0 (Molecular Devices, Union City, CA). SPs are characterized by a depolarizing phase (rising phase), the downward deflection, followed by a repolarizing phase (decline phase), the return to the baseline. After low-pass filtering (50 Hz, Gaussian filter),

Table 1 Composition of solutions used for single sensillum and patch-clamp recordings

	SSR recording electrode $[Ca^{2+}] = 6 \times 10^{-3}$ M	SSR recording electrode $[Ca^{2+}] = 10^{-3}$ M	SSR recording electrode $[Ca^{2+}] = 2 \times 10^{-8}$ M	SSR reference electrode	Patch recording electrode	Patch bath solution
KCl	172	172	172	6.4	150	4
Glucose	7.5	22.5	22.5	340	—	5
HEPES	10	10	10	10	10	10
$MgCl_2$	3	3	3	12	2	—
$CaCl_2$	6	1	0.02	1	1	6
NaCl	25	25	25	12	5	156
EGTA	—	—	4	—	11	—
Osmotic pressure (mOsm/l)	425 (glucose)	425 (glucose)	425 (glucose)	450 (glucose)	330 (mannitol)	360 (mannitol)
pH	6.5 (KOH)	6.5 (KOH)	6.5 (KOH)	6.5 (KOH)	7.2 (KOH)	7.2 (NaOH)

All concentrations are given in millimolars. The free Ca^{2+} concentration of 2×10^{-8} M was calculated with WebmaxC v.2.20. HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, EGTA, ethyleneglycol-bis(2-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetra-acetic acid.

3 different parameters were used to describe the rising phase (Figure 1). The SP latency was measured between the beginning of the stimulus as measured by the thermistor and the onset of the SP. The amplitude and halftime of rising phase ($t_{1/2\ rise}$) were measured as shown in Figure 1. The percentages of how much the SP had returned to the baseline after 800 ms and after 10 s were used to characterize the decline phase. APs were detected with Awave and counted in 100-ms bins for 20-s periods beginning 4 s before pheromone stimulation. The AP latency was measured between the onset of the SP and the positive peak of the first AP.

To record the spontaneous activity of ORNs, experiments were done on a second setup located in a different room where no pheromone compounds had ever been used to avoid any pheromone contamination. An Axopatch 200B amplifier (Molecular Devices) and a Digidata 1200A acquisition board (Molecular Devices) were used to record spontaneous activity onto a PC. The biological signal was amplified (×500) and low-pass filtered online (10 kHz). Then the signal was low-pass filtered offline (Gaussian, 50 Hz), and it was subtracted from the original trace to generate a pseudo high-pass filtering that does not distort the shape of APs (Dolzer et al. 2003). APs were detected from pseudo high-pass filtered traces and were counted and pooled in 10-s bins.

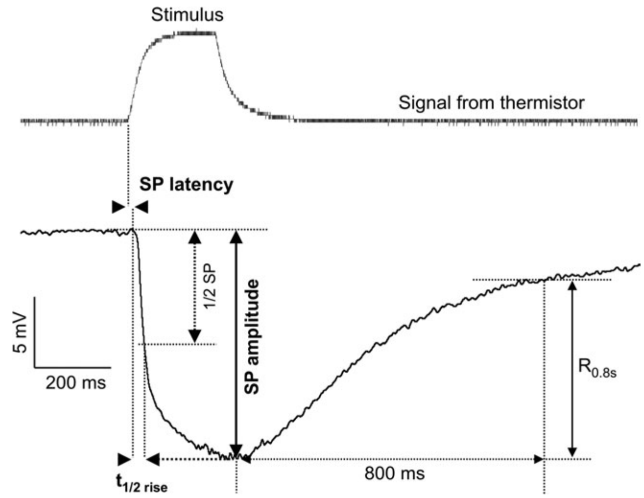

Figure 1 Parameters used to characterize SPs recorded in response to 500 ng of Z9,E11-14:Ac. SPs were filtered offline in Clampfit using a low-pass Gaussian filter at a cutoff frequency of 50 Hz. SP amplitude was measured between the baseline before the response and the negative peak during the response. SP latency is the time measured between the onset of the thermistor response and the onset of the SP. The halftime of the rising phase ($t_{1/2\ rise}$) is the time for the potential to reach half of SP amplitude. The potential was measured 800 ms ($R_{0.8s}$) and 10 s (R_{10s}) after SPs reached its maximal amplitude. For clarity only $R_{0.8s}$ is shown. The percentages of decline of the SP after 800 ms ($R_{0.8s}$/SP amplitude × 100) and 10 s (R_{10s}/SP amplitude × 100) were calculated.

Primary cell cultures

Whole-cell patch-clamp experiments were performed on ORNs grown in primary cultures from *S. littoralis* males. Cell cultures were prepared following the protocol previously reported (Lucas and Nagnan-Le Meillour 1997; Lucas and Shimahara 2002). Briefly, antennal flagella from 3-day-old male pupae were dissected. Cells were enzymatically and mechanically dissociated. The dispersed cells were plated onto uncoated Falcon Petri dishes in 3 parts of Leibovitz's L15 medium, 2 parts of Grace's medium supplemented with lactalbumin hydrolysate and yeastolate and conditioned on the embryonic cell line MRRL-CH1 (Eide et al. 1975), and 5% of fetal bovine serum (Invitrogen, Cergy Pontoise, France). Cultures were maintained in a humid atmosphere at 20 °C.

Patch-clamp recordings

Patch-clamp recordings closely followed the methods described in a previous paper (Lucas and Shimahara 2002). Recordings were performed at room temperature on neurons kept for 10–21 days in culture according to conventional patch-clamp methods (Hamill et al. 1981). Patch electrodes were pulled from thick-wall borosilicate capillaries (GC150-10, Harvard Apparatus, Les Ulis, France) using a horizontal P97 pipette puller (Sutter, Novato, CA). Electrodes had a tip resistance of 3.5–5 MΩ when filled with intracellular solution (Table 1). Currents were recorded with an Axopatch 200B amplifier (Molecular Devices) and digitized at 20 kHz using a Digidata 1322A (Molecular Devices) onto a PC. A holding potential of −60 mV was imposed on the membrane. Voltage steps of 100-ms duration from −80 to +100 mV in 10-mV increments were applied. Data were acquired and analyzed with pClamp 9.0 (Molecular Devices). A fractional (P/N) method, using 4 fractionally scaled hyperpolarized subpulses, was used for online leak compensation.

Statistics

We first used 2-way analysis of variance (ANOVA) to determine the overall significance for the time and Ca^{2+} effects on spontaneous activity and responses. When the ANOVA indicated a significant effect, a post hoc Tukey's multiple comparison of means test was used to determine individual differences between Ca^{2+} concentrations at each time and differences over the time for each Ca^{2+} concentration.

Results

To study the involvement of Ca^{2+} in ORN responses to pheromone stimuli in *S. littoralis*, the sensillum lymph was perfused with a high (6×10^{-3} M), an intermediate (10^{-3} M), and a low (2×10^{-8} M) Ca^{2+} concentration. The effects of the modification of the sensillar Ca^{2+} concentration were studied first on the TEP and the spontaneous firing activity and later on SP and firing responses to the pheromone stimuli.

The spontaneous firing activity depends on the sensillar Ca^{2+} concentration

Changing the sensillar Ca^{2+} concentration can potentially affect the physiology of accessory cells as well as ORNs. Because one of the functions of accessory cells is the control of the TEP with an electrogenic potassium pump located in their highly folded apical membrane (Küppers and Thurm 1979; Thurm and Wessel 1979), we checked whether changing the sensillar Ca^{2+} concentration affects the TEP. The mean TEP value was measured during the first and last 100 s of recordings lasting 35 min. Changes in Ca^{2+} concentration did not induce any drift in the TEP value. Mean differences between TEPs measured at the beginning and at the end of 35-min recordings did not differ significantly between recordings done at low (0.6 ± 8.7 mV, N = 11), intermediate (1.0 ± 7.4 mV, N = 9), and high (−1.9 ± 8.0 mV, N = 10) Ca^{2+} concentrations. We inferred from these data that the TEP does not depend on the sensillar Ca^{2+} concentration and that the Ca^{2+} effects described in this paper result from a direct effect on ORNs and not via an effect on accessory cells.

During the first 10 s of recording, the mean spontaneous firing activity did not depend significantly on the extracellular Ca^{2+} concentration with 0.3 ± 0.2 AP/s in low Ca^{2+}, 0.4 ± 0.6 AP/s in intermediate Ca^{2+}, and 0.5 ± 0.8 AP/s in high Ca^{2+} (Figure 2A). In intermediate and high Ca^{2+} concentrations, no significant difference in the mean spontaneous firing activity was found between the beginning (first 10 s) and the end (last 10 min) of recordings. In contrast, in low Ca^{2+} concentration the mean firing activity increased significantly. Between 25 and 35 min of recording, the firing activity was significantly higher ($P < 0.05$) in low Ca^{2+} (5.0 ± 4.3 AP/s) than in intermediate (1.9 ± 2.0 AP/s) and high (0.4 ± 0.7 AP/s) Ca^{2+}. A closer examination of the firing activity revealed that it was irregular in the low Ca^{2+} condition. Long bursts of APs associated with downward deflections of the TEP were only observed in low Ca^{2+} concentration.

Spontaneous downward deflections of the TEP had the same polarity as SPs recorded in response to odor stimuli, and they preceded a superimposed burst of APs that stopped immediately at the end of the odor stimuli. We called these TEP deflections spontaneous sensillar potentials (SSPs). To unambiguously separate SSPs from noise artifacts, only the duration and amplitude of SSPs larger than 0.5 mV were measured. SSPs were observed in the 3 Ca^{2+} conditions, but they were more frequent in low Ca^{2+} concentration. SSP frequency increased significantly ($P < 0.05$) from 0.3 ± 0.4 SSP/min in high and intermediate Ca^{2+} concentrations (N = 10 and 9 sensilla, respectively) to 1.3 ± 1.1 SSP/min (N = 11 sensilla) in low Ca^{2+} concentration. The duration of SSPs and the number of APs fired during an SSP were highly variable (Figure 2B). In low Ca^{2+} condition, SSPs lasted from 6 ms to 15.5 s, and the number of APs generated during a SSP ranged from 1 to 445. The duration of SSPs averaged 301 ± 1128 ms (N = 402) in low Ca^{2+}, 129 ± 177 ms (N = 92) in intermediate Ca^{2+}, and 80 ± 130 ms (N = 92) in high Ca^{2+}. There was no significant effect of the concentration of Ca^{2+} on SSP duration due to a high variability. However, the proportion of SSPs lasting more than 500 ms was higher in low Ca^{2+} (9.7%) compared with intermediate (2.2%) and high Ca^{2+} concentration (2.3%). The amplitude of SSPs did not vary with the concentration of Ca^{2+}. The average amplitude was 0.8 ± 0.6 mV (N = 92), 0.8 ± 0.4 mV (N = 88), and 0.8 ± 0.5 mV (N = 402) in high, intermediate, and low Ca^{2+} concentrations, respectively. Interestingly, in all 3

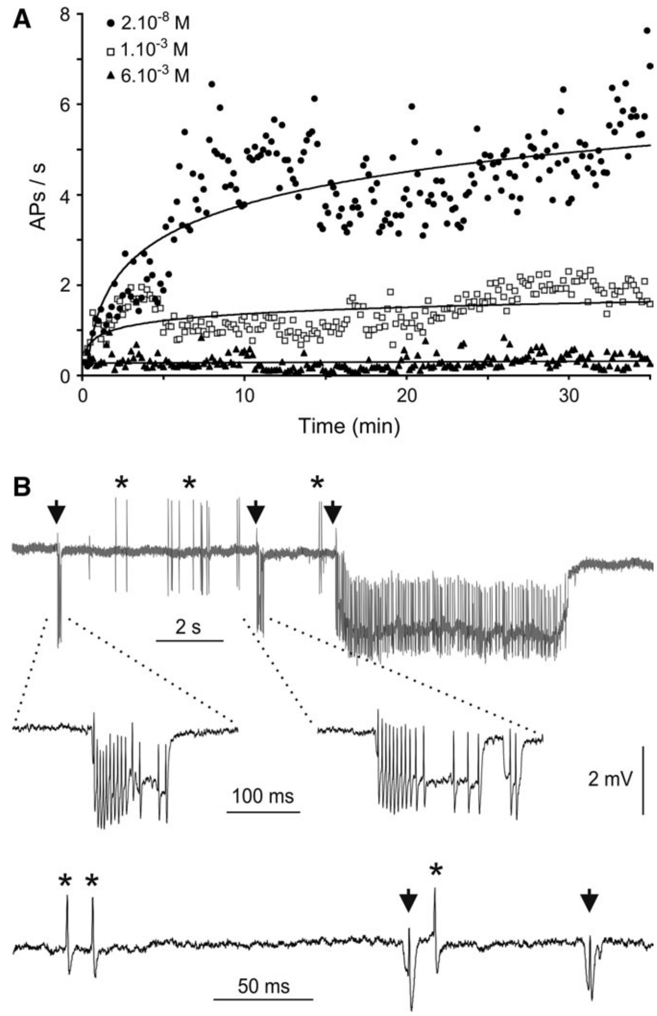

Figure 2 High sensillar Ca^{2+} concentration maintained the spontaneous firing activity at a low and regular frequency by stabilizing the membrane potential. **(A)** The average spontaneous firing activity was calculated from 35-min recordings and is expressed as the number of APs per second. Three different solutions differing in Ca^{2+} concentrations were used to fill the recording electrode: 2×10^{-8} M (N = 11), 10^{-3} M (N = 9), or 6×10^{-3} M (N = 10). **(B)** Unfiltered SSRs done with a recording electrode filled with a solution containing 2×10^{-8} M Ca^{2+}. In low Ca^{2+} condition, as in higher Ca^{2+} concentrations, APs were either preceded (arrows) or not (stars) by spontaneous negative deflections of the TEP that we called SSPs. The duration of SSPs was highly variable. Only in 2×10^{-8} M Ca^{2+}, SSPs lasting a few seconds with superimposed bursts of hundreds of APs were observed.

Ca^{2+} conditions, APs were generated both within SSPs (arrows, Figure 2B) and outside SSPs (asterisks, Figure 2B).

ORN response depends on intracellular but not on extracellular Ca^{2+} concentration

The ORN depolarization in response to pheromone stimulation does not depend on Ca^{2+} entry but depends on an increase in the intracellular Ca^{2+} concentration.

SPs and APs were recorded in response to the main pheromone component and compared in high, intermediate, and low Ca^{2+} concentrations. SP latency, SP amplitude, and $t_{1/2}$ rise did not depend on the Ca^{2+} concentration (Figure 3). Thus, SPs can be generated in low, intermediate, and high extracellular Ca^{2+} concentration without modification of the amplitude and kinetics of the SP rising phase.

We then checked if an intracellular source of Ca^{2+} is involved in ORN responses. In most cell types, changes in intracellular Ca^{2+} concentration are sensed by calmodulin, a signal transduction protein that regulates physiological target proteins. Because the intracellular Ca^{2+} concentration is difficult to manipulate, we perfused sensilla with a calmodulin antagonist, W-7, in low extracellular Ca^{2+} condition. In 5 out of 6 experiments, the perfusion with 10^{-4} M W-7 strongly reduced the SP amplitude and the number of APs fired in response to the pheromone stimulus after a delay of 11–21 min (Figure 4). After 31–41 min of perfusion with W-7, SPs and firing responses stabilized at about 30% of their initial amplitude. In contrast, responses remained at a constant level in control conditions.

SP decline depends on external Ca^{2+}

Decreasing extracellular Ca^{2+} concentration lengthened SP responses to pheromone stimulation (Figure 5A). The mean percentage of SP decline measured 800 ms after SP peak significantly differed between recordings made in high and low Ca^{2+} concentration (Figure 5B). The effect was more pronounced 10 s after SP peak. After 31 min of recordings, the mean value of SP decline differed significantly between the 3 Ca^{2+} treatments with 91% ± 18%, 68% ± 15%, and 32% ± 19% of SP decline in high, intermediate, and low Ca^{2+} concentration, respectively (Figure 5B). Thus, the kinetics of SP decline was negatively correlated to external Ca^{2+} concentration.

In high and intermediate Ca^{2+} concentrations, the SP decline had 2 phases, both of them exponential with respect to time but with different time constants, as shown in semilog plots (Figure 5C). The first phase occurred during the first 3 s after SP peak and was characterized by a fast decline, with time constants of τ_1 = 2.8 and 3.2 s at high and intermediate Ca^{2+}, respectively. The second phase presented a much slower decline with time constants of τ_2 = 10.4 and 25.8 s at high and intermediate Ca^{2+}, respectively. In low Ca^{2+} concentration, the time constant of the first phase of SP decline (τ_1 = 21.0 s) was much higher than in intermediate and high Ca^{2+}. In low Ca^{2+}, τ_1 was more similar to the time constant of the second phase of SP decline (τ_2 = 39.7 s), indicating that the first phase was abolished in low Ca^{2+} condition.

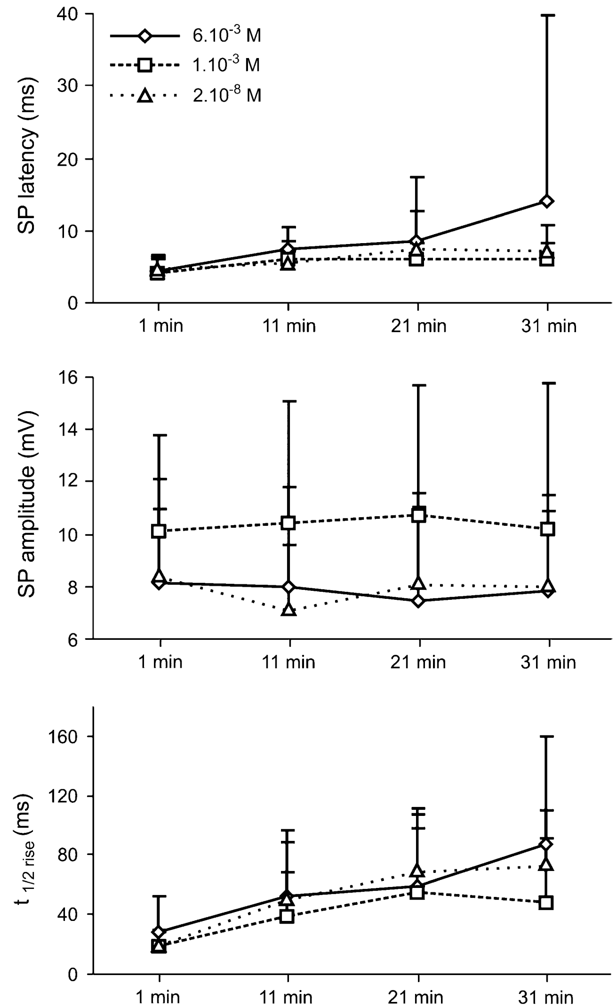

Figure 3 The rising phase of SPs did not depend on the concentrations of sensillar Ca^{2+}. Mean values of SP latency, SP amplitude, and $t_{1/2\ rise}$ were measured from responses to Z9,E11-14:Ac (500 ng, 200 ms) after 1, 11, 21, and 31 min of contact between the recording electrode and the sensillum. Three different solutions differing in the concentration of free Ca^{2+} were used to fill the recording electrode: 6×10^{-3} M (*N* = 10), 10^{-3} M (*N* = 9), or 2×10^{-8} M (*N* = 11). Error bars indicate standard deviation. No significant differences over time and between Ca^{2+} conditions were observed.

Delayed ORN repolarization affects firing responses

APs generated in response to pheromone stimuli were counted in 100-ms bins to establish mean poststimulus time histograms. The pheromone stimulus induced a transient increase in the firing activity that returned to the prestimulus level in 3–4 s (Figure 6A). The AP latency did not vary with the concentration of Ca^{2+} in the sensillar lymph (Figure 6B). Similarly, the amplitude of the response, taken as the number

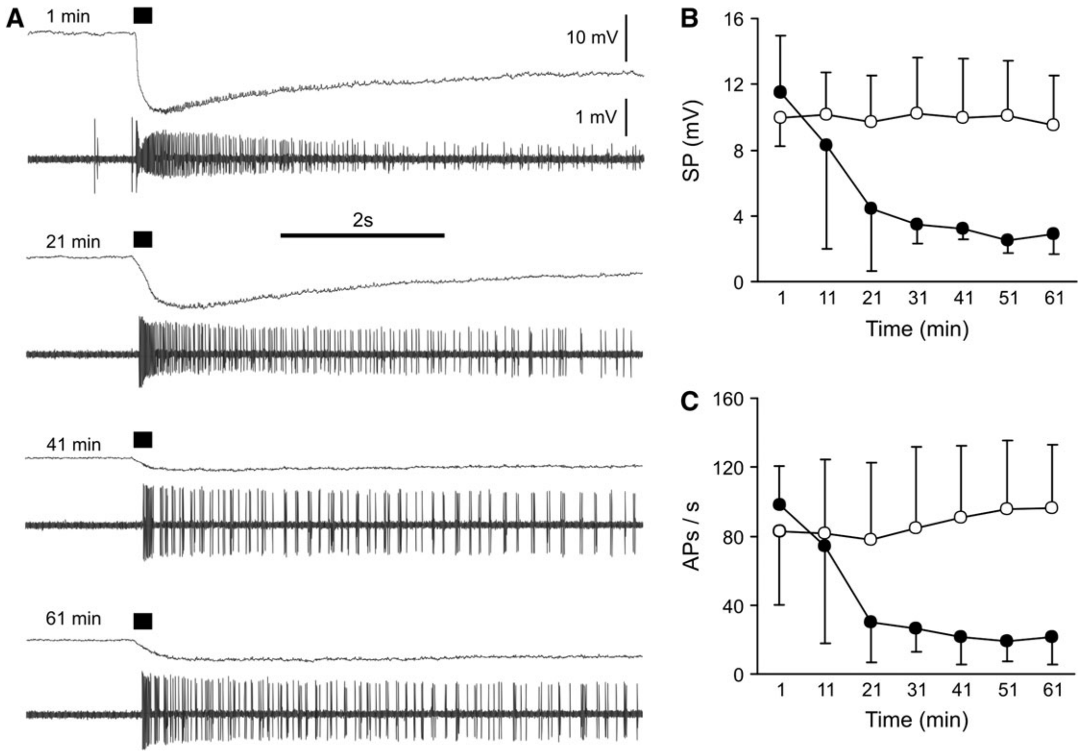

Figure 4 ORN responses depend on intracellular Ca^{2+} concentrations. **(A)** Responses to Z9,E11-14:Ac (10 ng, 200 ms) were recorded every 10 min from 1 to 61 min. Only responses recorded at 1, 21, 41, and 61 min are shown. At each time, the 2 traces above and below correspond to the low-pass filtered (DC to 50 Hz) and high-pass filtered (150–5000 Hz) signals, respectively. Black bars indicate the duration of pheromone stimulus. The recording electrode was filled with a solution containing 2×10^{-8} M Ca^{2+} and 100 μM W-7, a calmodulin antagonist. **(B)** Mean amplitude of SPs recorded in response to Z9,E11-14:Ac (10 ng, 200 ms) with 2×10^{-8} M of extracellular Ca^{2+} in presence (black circles, $N = 5$) or in absence (open circles, $N = 8$) of 100 μM W-7. **(C)** Mean number of APs fired during the first second following the beginning of the pheromone stimulus recorded with 2×10^{-8} M of extracellular Ca^{2+} and in presence (black circles, $N = 5$) or in absence (open circles, $N = 8$) of 100 μM W-7.

of APs fired during the 200-ms stimulation, was not affected by the extracellular Ca^{2+} concentration (Figure 6C).

After the response, the firing activity remained at a constant level in intermediate and high Ca^{2+}. In contrast, in the low Ca^{2+} condition, the firing activity was transiently inhibited for a few seconds. The mean number of APs fired between 6 and 10 s after the pheromone stimulus was significantly lower ($P < 0.05$) when recordings were done in low Ca^{2+} as compared with intermediate Ca^{2+} (Figure 6D). Thus, the delayed repolarization observed in low Ca^{2+} conditions induced an inhibition of the firing activity. Such an inhibition of the firing activity was also observed during long SPs obtained in response to strong or long pheromone stimuli in *S. littoralis* (data not shown).

ORNs express a voltage-dependent and Ca^{2+}-gated K^+ channel

ORN repolarization increased with extracellular Ca^{2+} concentration. This Ca^{2+} modulation of ORN repolarization can be either due to a downregulation of second messenger–dependent depolarizing channels and/or due to the activation of repolarizing currents. In *Mamestra brassicae* ORNs, a voltage- and Ca^{2+}-gated K^+ current ($I_{(KCa)}$) was characterized and is the main outward (repolarizing) current (Lucas and Shimahara 2002). Thus, we tested if such a current is also present in *S. littoralis* ORNs. Because ORNs are not readily accessible for patch-clamp recordings in situ, the search for $I_{(KCa)}$ was performed in cultured neurons.

In whole-cell voltage-clamp recordings, a sustained voltage-dependent outward current activated rapidly in response to depolarizing steps (Figure 7A). From a holding potential of −60 mV, this current appeared between −40 and −30 mV and became larger, to peak between +30 and +40 mV with amplitudes ranging from 409 to 755 pA (632 ± 137 pA, $N = 5$). The current to potential (I/V) curve of this sustained outward current always had an N shape (Figure 7C). Moreover, in the standard extracellular bath solution the N shape of the I/V curve faded spontaneously within a few minutes with a time course similar to the Ca^{2+} current rundown in *M. brassicae* ORNs (Lucas and Shimahara 2002), demonstrating the presence of a Ca^{2+}-dependent K^+ current. To isolate the Ca^{2+}-dependent component of the outward current, 10^{-3} M Co^{2+}, a blocker of Ca^{2+} channels, was added (Figure 7B). The subtracted Co^{2+}-sensitive outward current was a Ca^{2+}-dependent K^+

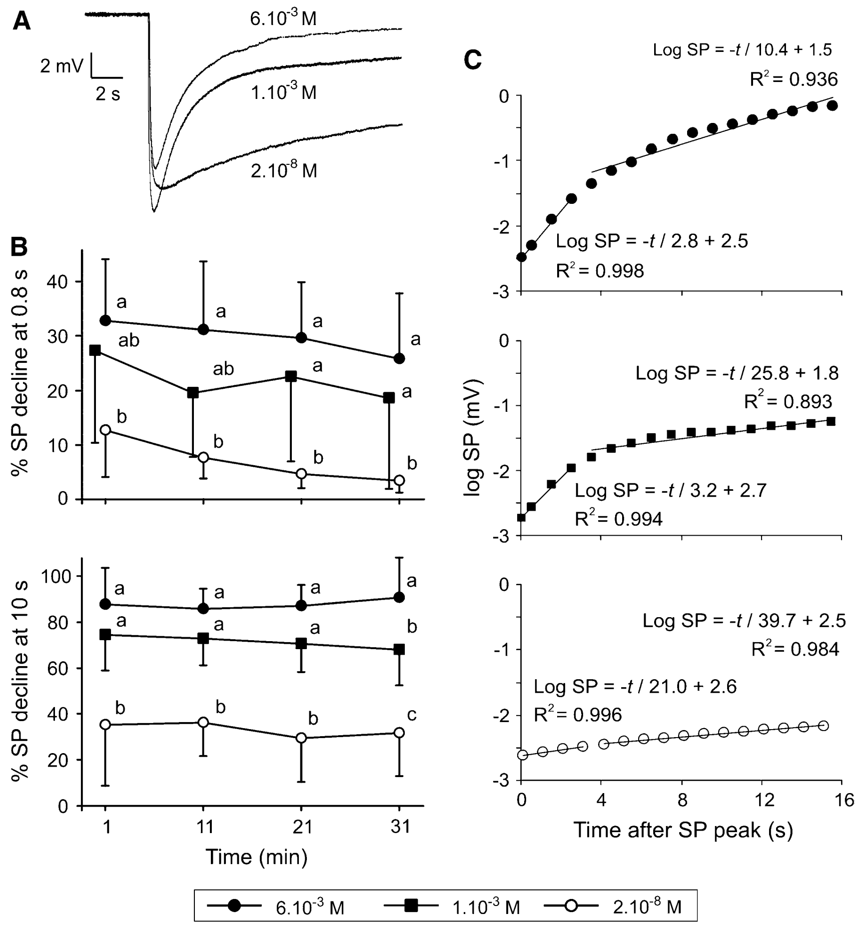

Figure 5 SP decline depended on the sensillar Ca^{2+} concentration. **(A)** Average traces made from SPs recorded in response to Z9,E11-14:Ac (500 ng, 200 ms) after 31 min of contact between the sensillum and the electrode. Solutions filling the recording electrode differed in their Ca^{2+} concentration: 2×10^{-8} M (N = 11), 10^{-3} M (N = 9), or 6×10^{-3} M (N = 10). **(B)** Percentages of SP decline at 0.8 and 10 s. Error bars indicate standard deviation. Symbols that share the same letter at a given time did not differ significantly ($P < 0.05$). **(C)** Semilog plots of the mean SP decline at 31 min, in high, intermediate, and low Ca^{2+} concentrations. The decline in SP has 2 phases. In each phase, the SP is an exponential function of time t of the form $SP = SP_{peak} \exp(-t/\tau)$ with SP_{peak} in millivolts and τ the time constant in seconds. In semilog plot log (SP) versus time t, the exponential curve is linearized as $\log SP = -t/\tau + \log SP_{peak}$.

current, which activated around −30 mV and reached a maximum of 455 ± 136 pA at +30 mV (N = 5).

Discussion

A combination of in vivo and in vitro electrophysiological recordings from *S. littoralis* ORNs provided insight into the role of extracellular and intracellular Ca^{2+} stores in moth olfactory transduction. Several new findings have emerged from this work. 1) The extracellular Ca^{2+} stabilizes the ORN membrane potential, and the spontaneous firing activity originates from mechanisms upstream of the spike generator site. 2) ORNs can depolarize in response to pheromone stimuli in low extracellular Ca^{2+}. 3) ORNs may contain a releasable pool of Ca^{2+} that participates in ORN responses. 4) ORN repolarization strongly depends on the extracellular Ca^{2+} concentration. Ca^{2+}-gated K^+ channels identified from cultured ORNs with whole-cell recordings are good candidates to mediate this ORN repolarization.

Ca^{2+} stabilizes the ORN membrane potential

In the 3 Ca^{2+} concentrations, we observed spontaneous downward deflections of the TEP that we called SSPs with superimposed APs. The SSPs we recorded from *S. littoralis* are reminiscent of elementary receptor potentials (ERPs) observed before each AP in the condition of weak pheromone stimulation in *Bombyx mori* and saturniid moths (Kaissling 1974; Redkozubov 1995; 2000b; Minor and Kaissling 2003). In *B. mori* and saturniid moths, ERPs last about 100 ms, and their amplitude reaches 200–300 μV. They were described as the primary electrical responses elicited by single-odor molecules (Kaissling 1987). Voltage-clamp recordings of elementary receptor currents revealed that a quantum event underlies ERPs (Redkozubov 2000b). ERPs apparently represent ORN depolarizations at the level of the outer dendrite

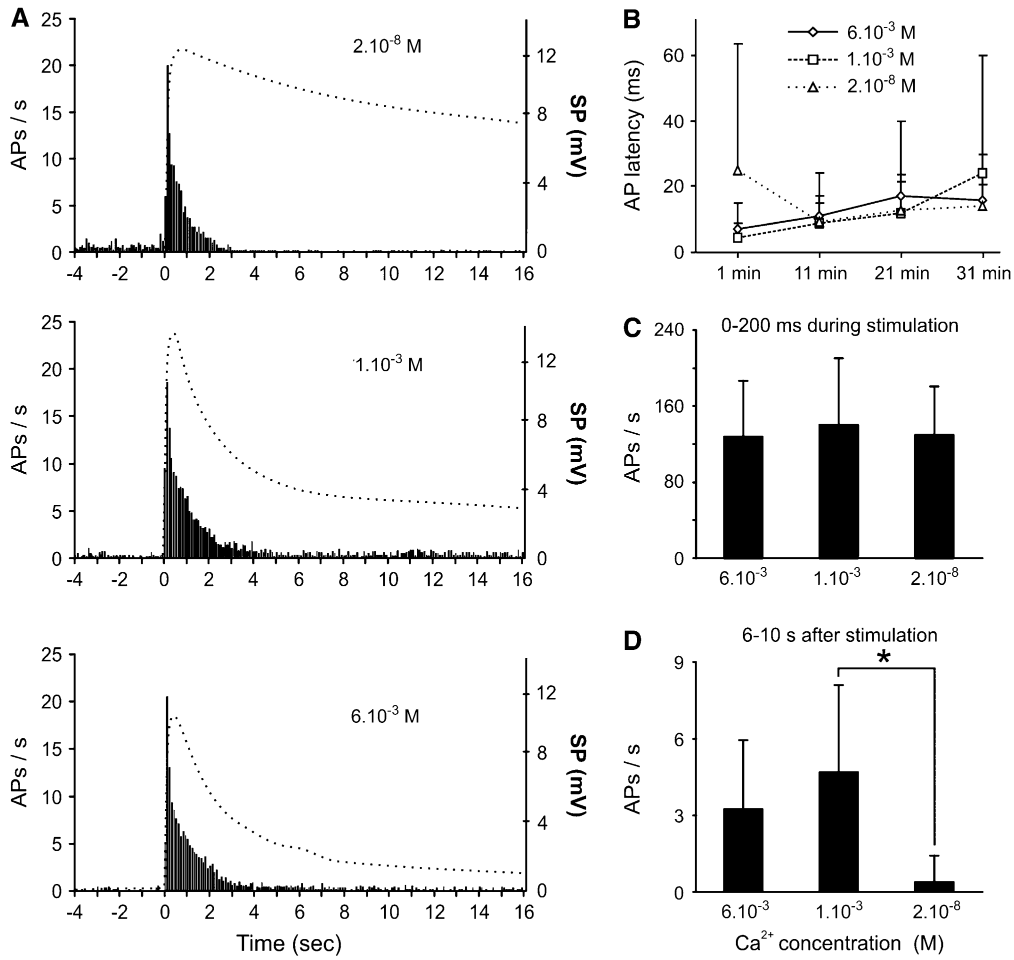

Figure 6 Kinetics of the AP discharge in response to Z9,E11-14:Ac (500 ng, 200 ms) recorded in 2×10^{-8} M ($N = 11$), 10^{-3} M ($N = 9$), or 6×10^{-3} M ($N = 11$) of extracellular Ca^{2+} concentration. **(A)** Mean poststimulus time histograms (PSTHs) established from response to pheromone stimulation recorded after 31 min of contact between the recording electrode and the sensillum. APs were counted per 100-ms bins from 4 s before to 16 s after the beginning of pheromone stimulus. Mean SPs from the same recordings are presented in broken lines upside down, as upward deflections of the potential, to have a visual comparison between SPs and PSTHs. **(B)** AP latency was measured between the onset of the SP and the positive peak of the first AP after 1, 11, 21, and 31 min of contact between the recording electrode and the sensillum. **(C)** Firing activity recorded 31 min after the contact between the recording electrode and the sensillum and measured during the 200-ms pheromone stimulus. **(D)** Firing activity recorded 31 min after the contact between the recording electrode and the sensillum and measured between 6 and 10 s after the pheromone stimulus. Asterisk indicates a significant difference between treatments ($P < 0.05$).

and could originate from the gating of single or clusters of channels as observed in *Drosophila* retinas (Haab et al. 2000).

SSPs were observed at all 3 Ca^{2+} concentrations tested. However, in intermediate or high Ca^{2+} concentration, SSPs were significantly less frequent and the proportion of long SSPs lasting more than 500 ms was lower than in low Ca^{2+} concentration. Moreover, the spontaneous firing activity was lower when sensillar Ca^{2+} was maintained at a high or an intermediate level. We propose that a Ca^{2+}-negative feedback regulates depolarizing currents that activate spontaneously. As a consequence of the Ca^{2+} feedback, fewer and shorter SSPs are generated, and unstimulated ORNs have a low and regular firing activity. Such feedback was observed in vitro in *M. sexta* ORNs on the Ca^{2+}-dependent cationic current that was activated after pheromone stimulation (Stengl 1994). We thus inferred from our data that Ca^{2+} stabilizes the membrane potential of ORNs.

Lastly, the low firing activity that we observed during the first seconds of recordings in all conditions of Ca^{2+} was better maintained in high than in intermediate Ca^{2+} concentration, suggesting that the physiological extracellular sensillar Ca^{2+} concentration in *S. littoralis* is closer to 6×10^{-3} M than to 10^{-3} M.

Spontaneous firing activity originates from noisy transduction mechanisms upstream of the spike generator site

Quantal-like current fluctuations similar to elementary receptor currents of *B. mori* were recorded from vertebrate ORNs and were interpreted as either being triggered by

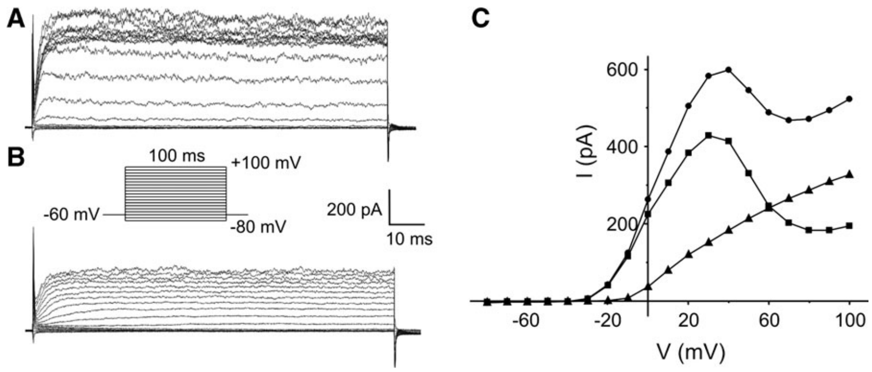

Figure 7 ORNs from *S. littoralis* expressed a voltage- and Ca^{2+}-gated K^+ current. **(A)** Voltage-clamp recording of the total outward current elicited by voltage steps from −80 to +100 mV in 10-mV increments of 100 ms. Holding potential was −60 mV. **(B)** Currents recorded from the same cell and the same voltage protocol as in (A) after the addition of 10^{-3} M Co^{2+} to block voltage-gated Ca^{2+} channels. **(C)** I/V curves from the total outward current shown in (A) (circles), the outward current that did not depend on the activation of voltage-gated Ca^{2+} channels shown in (B) (triangles), and the subtraction of (B) from (A), corresponding to the Ca^{2+}-dependent K^+ current (squares).

the binding of single-odorant molecules (Menini et al. 1995) or reflecting noise intrinsic to the transduction mechanism (Gold and Lowe 1995; Lowe and Gold 1995; Kleene 2000). To record true spontaneous activity and address the question of its origin, we recorded the electrical activity of ORNs on a new electrophysiological setup localized in a different room from the one used for recording pheromone responses. Even if we cannot totally exclude that some airborne pheromone molecules can have reached sensilla during recordings, the spontaneous firing activity we recorded most likely originated from noisy transduction mechanisms rather than weak pheromonal stimulations.

The sources of noise in the transduction process could come from any part of the biochemical cascade, from the production of second messengers to the activation of second messenger–gated channels generating ORN depolarization, and from the activation of voltage-gated channels involved in AP generation. During recordings of spontaneous ORN activity, spontaneous bursts of APs were superimposed on SSPs, particularly in low Ca^{2+} conditions. Bursts of APs always began after the onset of an SSP and never continued after its end, indicating that at least the firing activity during SSPs is the consequence of depolarizations upstream of the activation of voltage-dependent channels involved in AP generation. In *S. littoralis*, the inhibition of the degradation of DAG, using a DAG Kinase inhibitor, R59949, produced a sustained activation of a current that shares the properties of the DAG-activated current (Lucas and Pézier 2006). We interpreted this current as being due to constitutive PLC activity that leads to a DAG buildup, leading to activation of DAG-gated channels. The constitutive activity of PLC, leading to a basal biosynthesis of IP_3 and DAG, could be a source of spontaneous activity in insect ORNs.

In addition to APs fired during SSPs, at all Ca^{2+} concentrations, APs were also generated without any downward deflection of the TEP. This observation contrasts with recordings from *B. mori* where, in conditions of weak pheromone stimulation, all APs were generated following ERPs (Kaissling 1974, 1987) and brings into question whether APs generated without any preceding SSP originate from a different mechanism, e.g., spontaneous activation of voltage-gated channels. We consider it unlikely that the origin of spontaneous AP generation is located at the level of the spike generator because both the APs generated in the absence or during SSPs were generated at a frequency that depended on the extracellular Ca^{2+} concentration. In similar conditions of weak pheromone stimulation, ERPs are more difficult to discriminate from the noise in *S. littoralis* than in *B. mori* (Lucas P, personal observation). These observations suggest that not all SSPs could be discriminated from the noise due to their small amplitude and that all APs originate from a process upstream of spike generation.

ORNs can respond to pheromone stimuli in low extracellular Ca^{2+}

We then investigated the involvement of extracellular Ca^{2+} in ORN depolarization by analyzing the SP rising phase, which is considered to represent ORN depolarization (Vermeulen and Rospars 2001). It is generally agreed that in insects, the olfactory transduction cascade is mediated by G-protein-coupled receptors that activate PLC-β (Boekhoff, Raming, and Breer 1990; Boekhoff, Strotmann, et al. 1990; Boekhoff et al. 1993; Kalidas and Smith 2002), leading to the production of IP_3 and DAG. From patch-clamp recordings on cultured *M. sexta* ORNs, IP_3 was proposed to be the first second messenger of the pheromone transduction cascade, opening Ca^{2+} channels (Stengl 1994). This IP_3-dependent Ca^{2+} current precedes depolarizing cation currents. If depolarizing currents are strictly dependent on this IP_3-dependent Ca^{2+} entry, then low extracellular Ca^{2+} concentrations must reduce Ca^{2+} entry, leading to smaller receptor potentials. In contrast, in our recordings, SP amplitude, SP latency, and

$t_{1/2\ rise}$ did not differ between high, intermediate, and low Ca^{2+} concentration. The latency in the first AP generated and the number of APs fired during the stimulus also did not depend on the sensillar Ca^{2+} concentration. On the basis of our results, we conclude that the IP_3-dependent Ca^{2+} inward current is not strictly necessary for ORN depolarization.

To evaluate if Ca^{2+} release from intracellular stores is involved in ORN depolarization, we used W-7, a calmodulin antagonist. Calmodulin is an ubiquitous calcium-binding protein that can bind to and regulate a multitude of different protein targets, thereby affecting many different cellular functions. Calmodulin is a key component of the Ca^{2+} second-messenger system. Because ORNs can depolarize in response to pheromone even in low extracellular Ca^{2+}, we studied the effect of W-7 on responses to pheromone in low extracellular Ca^{2+} concentration. In the presence of W-7, the SP and the firing response to pheromone stimulations were strongly reduced but were not totally abolished, whereas responses remained stable in the control situation. Thus, intracellular Ca^{2+} appears to play a role in ORN depolarization. Two hypotheses, that are not mutually exclusive, can explain the responses observed in low extracellular Ca^{2+} concentration. As first suggested by Stengl (1994), IP_3 might not only cause an influx of extracellular Ca^{2+} through channels in the dendritic membrane but likely also release Ca^{2+} from intracellular stores as reported in many systems (Berridge 1993). The incomplete blocking effect of W-7 on pheromone response also suggests an additional mechanism of depolarization. A second transduction pathway based on DAG might be involved in the ORN response. DAG activates moth ORNs (Zufall and Hatt 1991; Redkozubov 1996; Pophof and Van Der Goes Van Naters 2002). Moreover, olfactory responses, but not adaptation, were normal in *Drosophila* mutants lacking IP_3-receptors (Deshpande et al. 2000), suggesting that IP_3 might not be required in the primary step of olfactory transduction in the fruit fly. The activation of DAG-gated cationic channels that we identified in *S. littoralis* ORNs (Lucas and Pézier 2006) could generate an additional depolarizing current independently of the extracellular Ca^{2+} concentration. Both the IP_3-dependent Ca^{2+} release leading to Ca^{2+}-dependent current activation and the DAG-gated current could co-localize and sustain depolarization independently of extracellular Ca^{2+} concentration.

Ca^{2+} is essential for the fast ORN repolarization

We investigated the role of Ca^{2+} in ORN repolarization by analyzing the SP decline, which represents the repolarization phase. In high and intermediate Ca^{2+}, the SP decline presented 2 steps, with a fast decline followed by a slower one. In contrast to the rising phase, the SP decline phase strongly depended on the concentration of Ca^{2+} in the sensillar lymph. The fast SP decline was abolished when extracellular Ca^{2+} was lowered to 2×10^{-8} M. The slower decline was less dependent on the extracellular Ca^{2+} concentration. These observations demonstrate that at least 2 different mechanisms underlie ORN repolarization, with an initial fast repolarization that depends heavily on the sensillar Ca^{2+} concentration followed by a slower repolarization that is less dependent on external Ca^{2+}.

ORN repolarization depends both on the termination of depolarizing currents and on the activation of repolarizing currents. Two cationic channels identified from cultured insect ORNs, one Ca^{2+}-gated (Stengl 1994, 1993) and the other DAG-activated (Lucas and Pézier 2006), are downregulated through a negative intracellular Ca^{2+} feedback. Both are thus Ca^{2+}-modulated channels that can generate long depolarizing currents and thus participate in the increase in the duration of SPs recorded in low sensillar Ca^{2+} condition.

With whole-cell patch-clamp recordings, we have identified a voltage- and Ca^{2+}-dependent K^+ current, $I_{K(Ca)}$, from *S. littoralis* ORNs. This current has fast kinetics of activation, and it is sustained. As in *M. brassicae* ORNs (Lucas and Shimahara 2002), $I_{K(Ca)}$ in *S. littoralis* is a voltage-dependent current with the largest amplitude in ORNs. Similar currents were identified from ORNs in *Locusta migratoria* (Wegener et al. 1992) and *M. sexta* (Dolzer 2002), but the channels underlying these currents remain to be identified. Beside their extraordinary sensitivity and selectivity, moth pheromone-responding ORNs are characterized by their strong temporal resolution (Willis and Baker 1984). The voltage and Ca^{2+} dependence of $I_{K(Ca)}$ and its rapid kinetics of activation are ideally suited for providing the fast repolarization of insect ORNs. The sensillum lymph that bathes the outer dendritic segment has an unusually high (200 mM) K^+ concentration (Kaissling and Thorson 1980). Thus, to be involved in repolarization, the K^+ channels must be located in membranes exposed to low external K^+ levels, such as the inner dendritic segment, the soma, or the axon.

As expected from the slower ORN repolarization observed in low Ca^{2+} condition, the firing activity recorded after pheromone stimuli depended on the external Ca^{2+} concentration. Only in low Ca^{2+} condition, did the ORN responses show an inhibition of the firing activity after an initial discharge of APs. Similar inhibitions of the firing activity were also observed in intermediate and high Ca^{2+} after responses to stimuli of the same intensity but longer duration. These inhibitions have been described as a mechanism of adaptation at the level of the spike generator (Zack and Kaissling 1986; Kaissling et al. 1987). Thus, the activation of voltage-gated current at the AP generator is independent of the Ca^{2+} concentration in the sensillum lymph. The firing inhibition observed under low Ca^{2+} concentration after pheromone responses appears to be a consequence of lengthened SPs in these conditions.

In conclusion, Ca^{2+} plays a key role in insect olfactory transduction. Ca^{2+} stabilizes the ORN membrane potential at rest, likely by a downregulation of channel openings gated by the spontaneous production of second messengers. This stabilization must confer a higher signal-to-noise ratio to the ORNs. Moreover, the fast termination of the response that is necessary for male orientation to calling females heavily depends on Ca^{2+}. Both Ca^{2+}-activated and Ca^{2+}-downregulated currents can account for the dependence of ORN repolarization on Ca^{2+}. Computational neurobiology based on quantitative analyses and modeling should allow us to clarify the respective importance of current modulation or activation on ORN repolarization.

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