Cyclic Nucleotide-Activated Currents in Cultured Olfactory Receptor Neurons of the Hawkmoth Manduca sexta

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Submitted 28 December 2008; accepted in final form 3 August 2008

Kranich S, Stengl M. Cyclic nucleotide-activated currents in cultured olfactory receptor neurons of the hawkmoth Manduca sexta. J Neurophysiol 100: 2866–2877, 2008. First published August 6, 2008; doi:10.1152/jn.01400.2007. Moth pheromones cause rises in intracellular Ca2+ concentrations that activate Ca2+-dependent cation channels in antennal olfactory receptor neurons. In addition, mechanisms of adaptation and sensitization depend on changes in cyclic nucleotide concentrations. Here, cyclic nucleotide-activated currents in cultured olfactory receptor neurons of the moth Manduca sexta are described, which share properties with currents through vertebrate cyclic nucleotide-gated channels. The cyclic nucleotide-activated currents of M. sexta carried Ca2+ and monovalent cations. They were directly activated by cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), modulated by Ca2+/calmodulin, and inhibited by lanthanum. M. sexta cyclic nucleotide-activated currents developed in an all-or-none manner, which suggests that the underlying channels are coupled and act coordinately. At least one cAMP- and two cGMP-activated nonselective cation currents could be distinguished. Compared with the cAMP-activated current, both cGMP-activated currents appeared to conduct more Ca2+ and showed a stronger down-regulation by Ca2+/calmodulin-dependent negative feedback. Furthermore, both cGMP-activated currents differed in their Ca2+-dependent inhibition. Thus M. sexta olfactory receptor neurons, like vertebrate sensory neurons, appear to express nonselective cyclic nucleotide-activated cation channels with different subunit compositions. Besides the nonselective cyclic nucleotide-activated cation currents, olfactory receptor neurons express a cAMP-dependent current. This current resembled a protein kinase-modulated low-voltage-activated Ca2+ current.

INTRODUCTION

Odors play a central role for the intra- and interspecific recognition and communication of insects. Male moths can detect almost single molecules of female sex pheromones (Kaißling and Priesner 1970). Accordingly, pheromone detection in moths is one of the best-studied models of how olfactory systems transduce odor information (Hansson 2002; Hildebrand 1995; Kaißling 2004; Rospars et al. 2007). Previous biochemical (Boekhoff et al. 1990; Breer et al. 1990), electrophysiological (Stengl 1993, 1994; Stengl et al. 1992; Wegener et al. 1997; Zufall and Hatt 1991; Zufall et al. 1991), and molecular genetic studies (Jacquin-Joly et al. 2002; Riego-Escobar et al. 1995) extensively characterized the initial steps of the insect olfactory transduction cascade. It is largely unknown, however, how pheromones induce adaptation or sensitization of the insect olfactory transduction cascade. Available data demonstrate that pheromone stimuli induce slow and delayed increases in the cyclic guanosine monophosphate (cGMP) concentration in the olfactory receptor neurons (ORNs) of different moth species (Boekhoff et al. 1993; Ziegelberger et al. 1990). Tip recordings from trichoid sensilla of moths showed that cGMP reduces the action potential frequency on pheromone stimulation and thus may trigger long-term adaptation (Flecke et al. 2006; Redkozubov 2000). The increase of cGMP after adapting pheromone stimuli appears to activate protein kinase G (Boekhoff et al. 1993). In single-channel recordings of Manduca sexta ORNs, millimolar concentrations of cGMP as well as cyclic adenosine monophosphate (cAMP) inhibited a pheromone-activated delayed rectifier potassium channel (Stengl et al. 1992; Zufall et al. 1991). Taken together, these findings suggest that cyclic nucleotides modulate olfactory transduction in moths.

In vertebrates, cyclic nucleotide-gated (CNG) channels have been extensively studied (Hofmann et al. 2005; Kaupp and Seifert 2002; Pifferi et al. 2006). Vertebrate olfactory CNG channels form heterotetrameric complexes composed of two principal CNGA2 subunits, a modulatory CNGB4, and a modulatory CNGB1b subunit (Bonigk et al. 1999; Sautter et al. 2001; Shapiro and Zagotta 1998; Shapero and Zagotta 1998; Zheng and Zagotta 2004). The subunit composition of vertebrate olfactory CNG channels determines their functional features such as ligand sensitivity, ion selectivity, and gating properties (Bradley et al. 2005; Munguer et al. 2001). Both cAMP and cGMP directly activate vertebrate olfactory CNG channels, which nonselectively conduct Ca2+ and monovalent cations (Dzeja et al. 1998; Frings et al. 1992, 1995). Since vertebrate olfactory CNG channels are negatively regulated by Ca2+/calmodulin (CaM), Ca2+ influx through CNG channels probably leads to adaptation (Bradley et al. 2001; Chen and Yau 1994; Munguer et al. 2001; Zufall and Leinders-Zufall 2000).

In insects, CNG channels have been characterized in the olfactory system of Drosophila melanogaster (Baumann et al. 1994; Miyazu et al. 2000), Apis mellifer (Gisselmann et al. 2003), and Heliothis virescens (Krieger et al. 1999). Here, we describe cyclic nucleotide-activated currents in the ORNs of M. sexta, which are likely to play a role for olfactory adaptation and sensitization. In whole cell patch-clamp recordings, we found that cAMP and cGMP directly activated currents. Pharmacological and ion exchange experiments demonstrated that at least one cAMP- and two cGMP-activated nonselective cation currents occurred in the ORNs of M. sexta. The cGMP-
activated currents carried more Ca$^{2+}$ and were more strongly influenced by Ca$^{2+}$/CaM-dependent negative feedback than the cAMP-activated current. Furthermore, the cGMP-activated currents differed in their Ca$^{2+}$-dependent inhibition. Unlike cGMP, cAMP activated a previously undescribed Ca$^{2+}$ current that resembled a protein kinase–modulated low-voltage–activated (LVA) Ca$^{2+}$ current.

METHODS

Cell culture media were obtained from Gibco (Karlsruhe, Germany) or PAA (PAA Laboratories, Colbe, Germany), reagents were purchased from Sigma (Taufkirchen, Germany), and salts for electrophysiological solutions were from Merck (Frankfurt am Main, Germany).

Insects

*M. sexta* (Johansson; Lepidoptera: Sphingidae) larvae were reared on an artificial diet (modified after Bell and Joachim 1976). Larvae were maintained under long-day photoperiod (L:D 17:7) at 24–27°C and 40–60% relative humidity. Pupae were staged using external markers (Jindra et al. 1997) and anesthetized by cooling until antennal flagella were dissected.

Primary cell cultures

The cell culture protocol was modified after Stengl and Hildebrand (1990). Briefly, antennae of male *M. sexta* pupae were dissected in Hanks’ balanced salt solution containing 1% penicillin–streptomycin solution (HBSS/PS). Antennae were incubated for 5 min at room temperature in HBSS/PS containing 1.3 mM EGTA, rinsed in HBSS/PS, and dissociated in two batches for 5 and 3 min at 37°C in HBSS/PS containing 24 mM papain. Dissociation was stopped with Leibovitz L-15 medium supplemented with 10% fetal bovine serum. The cell suspension was centrifuged at 90 to 110 relative centrifugal concentration, CaCl$_2$ in the extracellular solution was either reduced to 10$^{-5}$ or 10$^{-7}$ M (buffered with EGTA) or substituted with 6 mM BaCl$_2$. For simplicity, standard extracellular solution is referred to as “6Ca$^+$”; solutions with reduced calcium concentration as “low Ca”; and barium solution as “6Ba.” To identify voltage-gated channels, extracellular solutions contained 6 mM NiCl$_2$ (6Ni) or 1 mM ZnCl$_2$ (1Zn). The Cl$^-$-reduced extracellular solution (16 mM; “low Cl$^-$”) was obtained by replacing NaCl with the sodium acetate salt. A gravity-feed perfusion system controlled the exchange of extracellular solutions at a low flow rate. The complete exchange of extracellular solution took <1 min. The ionic composition of the patch pipette solution was identical in all experiments (in mM): CsCl 160; CaCl$_2$ 1; EGTA 11; and HEPES 10. Cesium was used to prevent potassium-dependent outward currents.

Reagents were directly pipetted into the extracellular solution. At the beginning of each experiment, extracellular solution was directly pipetted onto the cells as a control. After a delay of ≥2 min, cyclic nucleotides were applied. The membrane-permeant cAMP and cGMP analogues 8-bromo cAMP and 8-bromo cGMP were dissolved in extracellular solution and applied at final concentrations ranging from 5 nM to 50 µM (Supplemental Table S1). The calmodulin (CaM) antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and the nonspecific inhibitor of Ca$^{2+}$-permeable cation channels lanthanum (La$^{3+}$) were dissolved in water and applied at final concentrations of 10 to 50 µM for W-7 (Seno et al. 2005) and 0.05 to 1.1 mM for La$^{3+}$. The protein kinase inhibitors staurosporine (dissolved in dimethyl sulfoxide) and H7 (dissolved in EtOH) were applied at concentrations of 1 µM for staurosporine and 10 µM for H7 (Steng1 1993). Cell cultures were incubated with the respective protein kinase inhibitor for 15 to 30 min before breaking into whole cell configuration and application of cyclic nucleotides.

Patch-clamp technique and data analysis

Patch-clamp recordings were performed in whole cell configuration at room temperature according to the conventional patch-clamp method (Hamill et al. 1981). Cell cultures were monitored at 600× on an inverted microscope (Axioskope 135; Zeiss, Göttingen, Germany) equipped with phase-contrast optics. ORNs were identified on the basis of their morphology (Stengl and Hildebrand 1990). Patch electrodes were pulled from thick borosilicate glass capillaries (GC150T-10; Clark Electromedical Instruments, Reading, UK) with a micropipette puller (Sutter P97; Sutter Instruments, Novato, CA). Fire-polished patch pipettes with a tip resistance of 2 to 8 MΩ when filled with pipette solution were used to obtain seals of several gigaohms on the cell membrane. Junction potential was nullified prior to seal formation and capacitance and series resistance of the patch pipette were compensated. For whole cell recordings, the membrane potential was clamped at −60 mV. After breaking into whole cell configuration and a delay of ≥2 min for the stabilization of outward currents, the experiment was started. Three consecutive voltage-ramp protocols from −100 to +100 mV, with 500 ms each, were applied to establish current–voltage (I–V) relations.

Data acquisition was carried out with an Axopatch 1D amplifier using a Digidata 1200B interface (Molecular Devices, Union City, CA). Data acquisition and analyses were performed with pClamp (version 9; Molecular Devices). Currents recorded during voltage protocols were sampled at 20 kHz and low-pass filtered at 2 kHz. A MiniDigi acquisition device (MiniDigi1A; Molecular Devices) was used to continuously sample currents at 1 kHz and to record the time of drug application on a second acquisition channel. The figures show representative traces corrected for leak currents. The mean current amplitudes were determined at −100 mV. All data were presented as means ± SE. Statistical significance (P < 0.05) was evaluated by means of the two-tailed Student’s t-test. Increase of current amplitude was measured at ±100 mV and normalized to the maximum current amplitude obtained in control conditions. Inhibition of current amplitude was measured at ±100 mV and plotted as a percentage of the maximum current amplitude obtained under control conditions.

1 The online version of this article contains supplemental data.
RESULTS

Cyclic nucleotides activate currents in moth ORNs

To characterize cyclic nucleotide-activated currents, we stimulated *M. sexta* ORNs (*n* = 188) in whole cell patch-clamp recordings with the membrane-permeable cAMP and cGMP analogues 8-bromo cAMP and 8-bromo cGMP. Since a detailed analysis of all concentrations (5 nM to 50 μM) did not reveal statistically significant differences in the frequency of activation, current amplitude, reversal potential, and latency of activation over the total number of ORNs tested (Supplemental Table S1), we pooled the data.

Cyclic nucleotide application typically induced a stepwise increasing inward current with a delay of several seconds up to several minutes (cAMP 126 ± 9 s, *n* = 82 ORNs; cGMP 110 ± 10 s, *n* = 75 ORNs). The amplitude of the inward current typically reached a plateau and did not decline over the course of the recording (Fig. 1, A and B). Both cyclic nucleotides induced a current with a linear *I*–*V* relation in standard extracellular solution (6Ca; Table 1; Fig. 1, C and D). The cAMP-activated current had a mean reversal potential of 0.2 ± 1.6 mV and a mean amplitude of 608 ± 114 pA (*n* = 49 of 56 ORNs; Fig. 1C). The cGMP-activated current did not significantly differ from the cAMP-activated current and had a mean reversal potential of 1.6 ± 1.2 mV and a mean amplitude of 918 ± 208 pA (*n* = 43 of 52 ORNs; Fig. 1D). Application of La3+ (Table 2), a nonspecific blocker of Ca2+-permeable cation channels, significantly inhibited both cyclic nucleotide-activated currents (*P* < 0.01; Fig. 1), i.e., 71.6 ± 3.5% of the cAMP-activated current (*n* = 41 of 49 ORNs) and 68.1 ± 2.6% of the cGMP-activated current (*n* = 45 of 50 ORNs), respectively. The La3+-induced current inhibition did not significantly differ between cAMP- and cGMP-activated currents. However, La3+ inhibited the inward current of both cyclic nucleotide-activated currents significantly more strongly than the outward current (*P* < 0.01). The remaining La3+-independent outwardly rectified current had a mean reversal potential of −5.4 ± 2 mV for cAMP- and −1.8 ± 1.5 mV for cGMP-activated currents.

Both cyclic nucleotide-activated currents showed a La3+ concentration dependence (Fig. 1, A, B, E, and F). Low-La3+...
concentrations (50 to 100 μM) inhibited cAMP-activated currents in about 29% of ORNs, but potentiated them in about 53% of ORNs (n = 17 applications). High-La\(^{3+}\) concentrations (0.13 to 1.1 mM) inhibited cAMP-activated currents in about 84% of ORNs and potentiated them in only about 9% of ORNs (n = 45 applications; Fig. 1E). Similarly, low-La\(^{3+}\) concentrations inhibited cGMP-activated currents in approximately 43% of ORNs and potentiated them in about 29% of ORNs (n = 14 applications). High-La\(^{3+}\) concentrations inhibited the cGMP-activated current in about 91% of ORNs and potentiated them in only about 7% of ORNs (n = 45 applications; Fig. 1F). In the remaining ORNs, La\(^{3+}\) had no effect.

**Cyclic nucleotides activate nonselective cation currents**

To investigate whether cAMP- and cGMP-activated currents depend on the extracellular Ca\(^{2+}\) concentration, CaCl\(_2\) was reduced in the extracellular solution to either 10 \(-5\) or 10 \(-7\) M (low Ca) or substituted with 6 mM BaCl\(_2\) (6Ba; Table 1). Both cAMP and cGMP induced currents in low-Ca and 6Ba solutions. Subsequent La\(^{3+}\) application inhibited these currents. In low-Ca solution [see Fig. 4, A–C, traces (I)], cAMP induced a linear or inwardly rectified current with a mean reversal potential of \(-0.5 \pm 4.3\) mV and a mean amplitude of 1,001 ± 391 pA (n = 13 of 17 ORNs). Similarly, the cGMP-activated current showed a linear \(I–V\) relation, a mean reversal potential of 6.8 ± 4.9 mV, and a mean amplitude of 997 ± 280 pA (n = 13 of 16 ORNs). In 6Ba solution, both cyclic nucleotides induced currents with a linear \(I–V\) relation. The cAMP-activated current (Fig. 2A) had a mean reversal potential of \(-0.4 \pm 1.6\) mV and a mean amplitude of 749 ± 199 pA (n = 6 ORNs). The cGMP-activated current (Fig. 2B) had a mean reversal potential of \(-3.6 \pm 3.1\) mV and a mean amplitude of 1,054 ± 719 pA (n = 7 of 8 ORNs). The mean amplitudes or reversal potentials of the cAMP- and cGMP-activated currents did not significantly differ in the various extracellular solutions. In some experiments (n = 8 of 14 ORNs), 6Ba solution induced a small inward current through Ca\(^{2+}\) channels (Fig. 2B).

To examine whether K\(^+\) and Na\(^+\) equally contribute to the cAMP- and cGMP-activated currents (cAMP n = 5 ORNs; cGMP n = 3 ORNs; data not shown), cyclic nucleotide-activated currents were recorded with standard Cs\(^+\) pipette solution (160 mM) and successively exposed to extracellular solution containing 160 mM Cs\(^+\), K\(^+\), and Na\(^+\), respectively. The conductances for outward currents were measured relative to Cs\(^+\) at +100 mV. The conductances of the cAMP-activated current were: K\(^+\) (1.3):Na\(^+\) (1.2):Cs\(^+\) (1); those of the cGMP-activated current were: K\(^+\) (1.2):Na\(^+\) (1) ~ Cs\(^+\) (1).

**Cyclic nucleotide-activated currents depend on the extracellular Ca\(^{2+}\) concentration**

To investigate the Ca\(^{2+}\) dependence of cyclic nucleotide-activated currents, ORNs were exposed to different extracellular Ca\(^{2+}\) concentrations. In a first set of experiments, ORNs were initially kept in 6Ca solution, stimulated with cyclic nucleotides, and then perfused with low-Ca solution (10 \(-5\) M; Table 2). Both the cAMP-activated (n = 8 ORNs; Fig. 3A) and the cGMP-activated (n = 5 of 7 ORNs; Fig. 3B) currents significantly increased in low-Ca solution (\(P < 0.01\); Fig. 3C). Subsequent La\(^{3+}\) application inhibited the cyclic nucleotide-activated currents (Fig. 3). After perfusion with low-Ca solution, both cyclic nucleotide-activated currents expressed a linear \(I–V\) relation with a constant mean reversal potential.

In a second set of experiments, ORNs were at first kept in low-Ca solution, stimulated with cyclic nucleotides, and then perfused with 6Ca solution (Table 2). In 5 of 7 ORNs, perfusion with 6Ca solution significantly inhibited 35 ± 4% of the cAMP-activated current (\(P < 0.01\); Fig. 4, A and D). The mean reversal potential of the linear or outwardly rectified cAMP-activated current remained unchanged. The cGMP-activated current showed a comparatively complex Ca\(^{2+}\) dependence. In general, the cGMP-activated current significantly increased after perfusion with 6Ca solution (\(P < 0.05\); n = 6 of 9 ORNs; Fig. 4, C and E). However, in 3 of 9 ORNs, perfusion with 6Ca solution significantly inhibited 56.5 ± 7.9% of the cGMP-activated current (\(P < 0.01\); Fig. 4, B and D). The cGMP-activated current showed a significantly stronger current inhibition than that of the cAMP-activated current (\(P < 0.05\)) in 6Ca solution. The mean reversal potential of the linear cGMP-activated currents remained unchanged. Following perfusion with 6Ca solution, La\(^{3+}\) application further inhibited 61 ± 11.7% of the remaining cyclic nucleotide-activated currents (n = 3 ORNs; data not shown), but did not significantly change the mean reversal potential.

**Cyclic nucleotide-activated currents are modulated by Ca\(^{2+}\)/CaM**

To test whether Ca\(^{2+}\)/CaM inhibits cyclic nucleotide-activated currents, the CaM antagonist W-7 was applied. Application of W-7 (10 to 50 μM) alone did not elicit a current response (n = 14 ORNs), but significantly increased the cyclic nucleotide-activated currents (Table 2). Application of W-7 induced an approximately twofold increase of the cAMP-activated current (\(P < 0.01\); n = 8 of 12 ORNs; Fig. 5, A and C) and an almost tenfold increase of the cGMP-activated current (\(P < 0.05\); n = 7 of 14 ORNs; Fig. 5, B and C). Thus

### Table 1. Cyclic nucleotide-activated currents in moth ORNs

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<tr>
<th></th>
<th>cAMP</th>
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<tbody>
<tr>
<td>6Ca</td>
<td>56</td>
<td>52</td>
<td>88</td>
<td>83</td>
<td>linear</td>
<td>linear</td>
<td>0.2 ± 1.6</td>
<td>1.6 ± 1.2</td>
</tr>
<tr>
<td>+ Stauroporine</td>
<td>8</td>
<td>9</td>
<td>88*</td>
<td>67</td>
<td>linear</td>
<td>linear</td>
<td>5.7 ± 2.3</td>
<td>8.0 ± 8.7</td>
</tr>
<tr>
<td>+ H7</td>
<td>9</td>
<td>7</td>
<td>67*</td>
<td>86</td>
<td>linear/inward</td>
<td>inward</td>
<td>5.7 ± 3.6</td>
<td>5.3 ± 3.3</td>
</tr>
<tr>
<td>6Ba</td>
<td>6</td>
<td>8</td>
<td>100</td>
<td>88</td>
<td>linear</td>
<td>linear</td>
<td>0.4 ± 1.6</td>
<td>3.6 ± 3.1</td>
</tr>
<tr>
<td>Low Ca</td>
<td>17</td>
<td>16</td>
<td>77</td>
<td>81</td>
<td>linear/inward</td>
<td>linear</td>
<td>0.5 ± 4.3</td>
<td>6.8 ± 4.9</td>
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</table>

Values are means ± SE; n represents number of ORNs recorded; +, incubation with protein kinase inhibitor in 6Ca; *except activation of \(I_{\text{cGMP}}\). See METHODS for solutions.

W-7 increased the cGMP-activated current significantly more strongly than the cAMP-activated current (P < 0.05; Fig. 5C). Apart from the differing current amplitudes, the properties of the cyclic nucleotide-activated currents remained unchanged, i.e., the currents still showed a linear I–V relation, a mean reversal potential of −0.5 ± 1.7 mV for cAMP- and 1.2 ± 4.3 mV for cGMP-activated currents, and inhibition by La3⁺.

**Ni²⁺ and Zn²⁺ inhibit cyclic nucleotide-activated currents**

Transition metals like Ni²⁺ and Zn²⁺ inhibit Ca²⁺ permeable channels. Thus to further characterize the Ca²⁺ dependence of cyclic nucleotide-activated currents, ORNs were kept in 6Ca solution, stimulated with cyclic nucleotides, and then exposed to solutions containing 6 mM NiCl₂ (6Ni) or 1 mM ZnCl₂ (1Zn; Table 2). Both the 6Ni and 1Zn solution significantly reduced the cAMP- and the cGMP-activated currents (P < 0.01). Perfusion with 6Ni solution inhibited 56 ± 7.3% of the cAMP-activated current (n = 7 of 13 ORNs, Fig. 6), whereas 1Zn solution inhibited 31.1 ± 5.4% (n = 5 of 7 ORNs; Fig. 6). Thus the 6Ni solution induced a significantly stronger current decline than the 1Zn solution (P < 0.05). The mean reversal potential of the outwardly rectified currents remained unchanged in both solutions. Similarly, perfusion with 6Ni solution inhibited 57.3 ± 8% of the cGMP-activated current (n = 9 of 14 ORNs, Fig. 6) and 1Zn solution inhibited 40.9 ± 7.6% (n = 6 ORNs; Fig. 6). In contrast to the cAMP-activated current, the cGMP-activated current appeared to be equally inhibited by the 6Ni and 1Zn solutions. The mean reversal potential of the outwardly rectified currents remained unchanged in both solutions. La³⁺ inhibited 61.5 ± 6.3% of the remaining cyclic nucleotide-activated currents in 6Ni solution (n = 13 of 16 ORNs) and 72.3 ± 7.1% in 1Zn solution (n = 5 of 6 ORNs; data not shown), but did not significantly change the mean reversal potential. In some experiments, however, perfusion with 6Ni solution increased cAMP- (n = 4 of 13 ORNs) or cGMP-activated currents (n = 3 of 14 ORNs; data not shown).

**Cyclic nucleotide-activated currents are modulated by extracellular Cl⁻**

To investigate whether Cl⁻ currents constitute a fraction of the cyclic nucleotide-activated currents, ORNs were kept in 6Ca solution, stimulated with cyclic nucleotides, and then exposed to an extracellular solution with a reduced Cl⁻ concentration (low Cl; Table 2). In 6Ca solution, cAMP-activated currents had a mean reversal potential of −0.2 ± 3 mV, which significantly shifted to −11.6 ± 4 mV in low-Cl solution (P < 0.05; n = 9 ORNs). Similarly, the mean reversal potential of cGMP-activated currents significantly shifted from 7.1 ± 8 mV in 6Ca solution to −14.3 ± 3.9 mV in low-Cl solution (P < 0.05; n = 7 ORNs). Perfusion with low-Cl solution typically increased both the cAMP- (n = 5 of 9 ORNs; Fig. 7A) and the cGMP-activated currents (n = 5 of 7 ORNs; Fig. 7B). Subsequent La³⁺ application inhibited 82.9 ± 4% of the cyclic nucleotide-activated currents in low-Cl solution (n = 10 ORNs; Fig. 7), but did not significantly change the mean reversal potential.

**Cyclic nucleotides activate currents independently of protein kinases**

To investigate whether cAMP- and cGMP-activated currents depend on protein kinases, ORNs were preinhibited with the protein kinase inhibitors staurosporine (1 μM) or H7 (10 μM) for ≥15 min and then stimulated with cyclic nucleotides. Both protein kinase inhibitors did not prevent cAMP- and cGMP-activated currents. However, staurosporine sometimes induced a small inward current (control; I) through Ca²⁺ channels (B).

**TABLE 2. Pharmacological properties of cyclic nucleotide-activated currents**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Inhibition, %</th>
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<td>cAMP</td>
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<tr>
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<td>0</td>
<td>14</td>
<td>67</td>
</tr>
<tr>
<td>++ La³⁺</td>
<td>49</td>
<td>50</td>
<td>83</td>
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<td>11</td>
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<td>8</td>
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<td>0</td>
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<tr>
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<td>9</td>
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<td>54</td>
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<tr>
<td>&gt; 1Zn</td>
<td>7</td>
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<tr>
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<td>9</td>
<td>7</td>
<td>11</td>
<td>14</td>
<td>56</td>
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</tbody>
</table>

Values are means ± SE; n represents number of ORNs recorded; ++, bath application, >, perfusion. Remaining ORNs showed no effect. See METHODS for solutions.
activated nonselective cation currents (Table 1). In the presence of staurosporine, both cyclic nucleotide-activated currents showed a linear $I$-$V$ relation (Fig. 8, A and B). The cAMP-activated current had a mean reversal potential of $5.7 \pm 2.3$ mV and a mean amplitude of $607 \pm 343$ pA ($n = 7$ of 8 ORNs; Fig. 8A). Comparably, the cGMP-activated current had a mean reversal potential of $738 \pm 253$ pA ($n = 6$ of 9 ORNs; Fig. 8B). In the presence of H7, both cyclic nucleotides predominantly activated an inwardly rectified current (Fig. 8, C and D). The cAMP-activated current had a mean reversal potential of $5.7 \pm 3.6$ mV and a mean amplitude of $330 \pm 243$ pA ($n = 6$ of 9 ORNs; Fig. 8C). Similarly, the cGMP-activated current had a mean reversal potential of $5.3 \pm 3.3$ mV and a mean amplitude of $142 \pm 55$ pA ($n = 6$ of 7 ORNs; Fig. 8D). Subsequent La$^{3+}$ application inhibited both cyclic nucleotide-activated currents.

Unlike cGMP, cAMP activated an additional inward current in the presence of H7 and staurosporine. This current immediately appeared after cAMP application and showed the typical $I$-$V$ relation of an LVA Ca$^{2+}$ current, activating at about $-60$ mV and a peak at about $-20$ mV (Fig. 8C). The cAMP-activated Ca$^{2+}$ inward current [$I_{Ca(cAMP)}$] declined within seconds to minutes or was superimposed by the cAMP-activated nonselective cation current. In the presence of H7, the $I_{Ca(cAMP)}$ had a mean amplitude of $-39.8 \pm 9.1$ pA and a mean reversal potential of $44.3 \pm 8.3$ mV ($n = 8$ of 9 ORNs). Three ORNs exclusively showed the $I_{Ca(cAMP)}$. In the presence of staurosporine, cAMP application induced the $I_{Ca(cAMP)}$ in 4 of 7 ORNs (data not shown). In these ORNs, the $I_{Ca(cAMP)}$ had a mean amplitude of $-11.3 \pm 0.3$ pA and a mean reversal potential of $14.3 \pm 8.8$ mV. One ORN exclusively showed the $I_{Ca(cAMP)}$.

**DISCUSSION**

We used whole cell patch-clamp recordings to characterize cyclic nucleotide-activated currents in cultured ORNs of *M. sexta* that are possibly involved in olfactory adaptation and sensitization. Pharmacological and ion-exchange experiments distinguished at least one cAMP- and two cGMP-activated nonselective cation currents, which significantly differed in their Ca$^{2+}$ dependence and Ca$^{2+}$/CaM-dependent inhibition. Furthermore, a cAMP-dependent LVA Ca$^{2+}$ current was described for the first time that was modulated by protein kinases.

**Moth ORNs appear to possess CNG channels**

In *M. sexta* ORNs, cyclic nucleotide-activated currents have properties similar to those of currents through vertebrate olfactory CNG channels. Since cGMP has been shown to activate a 55-pS nonselective cation channel in single-channel recordings (Dolzer et al. 2008), our findings suggest that cyclic nucleotide-activated currents depend on prospective *M. sexta* cyclic nucleotide-gated (MsCNG) channels. Like vertebrate olfactory CNG channels (Hofmann et al. 2005; Kaupp and Seifert 2002; Pifferi et al. 2006), the prospective MsCNG channels are Ca$^{2+}$ permeable nonselective cation channels that are directly activated by both cAMP and cGMP. Both prospective MsCNG channels and vertebrate olfactory CNG channels show weak voltage dependence and do not desensitize in the continuous presence of cyclic nucleotides. In addition, prospective MsCNG channels and vertebrate olfactory CNG channels are modulated by Ca$^{2+}$/CaM. Like vertebrate olfactory CNG channels, the prospective MsCNG channels differ in ligand sensitivity, ion selectivity, and gating properties.
Unlike vertebrate olfactory CNG channels, however, prospective MsCNG channels did not change their response to varying concentrations of cAMP and cGMP. This lack of dose dependence was also found for cGMP-activated channels in single-channel recordings of *M. sexta* ORNs (Dolzer et al. 2008). Since both cAMP and cGMP typically induced a stepwise inward current, prospective MsCNG channels are probably coupled and act coordinately. Accordingly, in single-channel recordings of *M. sexta* ORNs, cGMP-dependent large-conductance events consisted of several simultaneous subconductance events in the open state (Dolzer et al. 2008). In the rat olfactory system, the principal CNG channel subunit CNGA2 localizes to lipid rafts (Brady et al. 2004) that facilitate the lateral assembly of signaling cascades (Simons and Toomre 2000) and thus likely coordinate gating. Compartmentalization of vertebrate olfactory CNG channels ensures rapid and efficient cyclic nucleotide signaling (Brady et al. 2004). Since prospective MsCNG channels appear to open in a concerted manner, several lipid rafts possibly congregate prospective MsCNG channels in the cell membrane of ORNs and act coordinately.

**Prospective MsCNG channels differ in their Ca$^{2+}$ dependence**

Like homomeric olfactory CNG channels (Frings et al. 1995; Seifert et al. 1999), prospective MsCNG channels differ in their sensitivity to extracellular Ca$^{2+}$. Increases of extracellular Ca$^{2+}$ inhibited the cAMP-activated currents and one type of the cGMP-activated currents. This is in accordance with the
finding that high extracellular Ca\(^{2+}\)/CaM concentrations generally inhibit monovalent cation currents and turn vertebrate olfactory CNG channels into “pure” Ca\(^{2+}\)/CaM channels (Dzeja et al. 1999; Frings et al. 1995). Importantly, Ca\(^{2+}\)/CaM significantly more strongly inhibited cGMP-activated currents than cAMP-activated currents. Since the molecular structure of the channel pore determines the Ca\(^{2+}\)/CaM permeability of vertebrate CNG channels (Eismann et al. 1994; Root and MacKinnon 1993; Seifert et al. 1999; Sesti et al. 1995), the prospective cAMP- and cGMP-dependent MsCNG channels are likely to differ in their pore region. Besides the inhibitory effect of Ca\(^{2+}\)/CaM on cAMP- and cGMP-activated currents, Ca\(^{2+}\) also increased the current through at least one additional cGMP-dependent prospective MsCNG channel. So far it is unknown which ORNs express which specific prospective MsCNG channel types. Since ORNs do not differ in their morphology in primary cell cultures (Stengl and Hildebrand 1990), we grouped ORNs into different subpopulations according to their electrophysiological properties. Because the increase of external Ca\(^{2+}\) induced opposite effects on cGMP-activated currents, at least two types of prospective cGMP-dependent MsCNG channels appear to be expressed in specific ORNs.

**Prospective MsCNG channels differ in their Ca\(^{2+}\)/CaM-dependent inhibition**

Cyclic nucleotides do not directly inactivate vertebrate olfactory CNG channels, but instead induce adaptation of the respective channels via Ca\(^{2+}\)/CaM-dependent negative feedback (Bradley et al. 2005; Chen and Yau 1994; Trudeau and Zagotta 2003). In *M. sexta* ORNs, the CaM antagonist W-7 increased the amplitude of cyclic nucleotide-activated currents. Thus Ca\(^{2+}\)/CaM obviously provides negative feedback to these currents in the continuous presence of cyclic nucleotides. In the vertebrate olfactory system, native CNG channels are composed of two CNGA2, one CNGA4, and one CNGB1b subunit (Bonigk et al. 1999; Sautter et al. 1998; Shapiro and Zagotta 1998; Zheng and Zagotta 2004). The CNGA2 subunit contains a CaM binding site and controls gating (Liu et al. 1994), whereas the modulatory subunits CNGA4 and CNGB1b contain IQ-motifs that regulate the kinetics of Ca\(^{2+}\)/CaM-mediated inhibition. Native CNG channels are rapidly inhibited by Ca\(^{2+}\)/CaM. However, vertebrate olfactory CNG channels, which consist of
only CNGA2 subunits or two differing subunits (CNGA2 +
CNGA4/B1b), show a distinctively reduced current decline
due to a reduced rate of Ca\(^2+\)/CaM binding to the CNGA2
subunit (Bradley et al. 2001, 2004; Munger et al. 2001).
Since W-7 induced a stronger activation of the cGMP- than
the cAMP-activated currents in \textit{M. sexta} ORNs, the corre-
sponding prospective MsCNG channels may differ in their
subunit composition.

\textit{Lanthanum and transition metals inhibit prospective
MsCNG channels}

Several studies used La\(^{3+}\) to inhibit Ca\(^{2+}\)-permeable cation
channels such as TRP channels (Clapham et al. 2005), Ca\(^{2+}\)-
avtivated chloride channels (Tokimasa and North 1996), or store-
operated Ca\(^{2+}\) channels (\textit{l}_{\text{CRAC}}; Hoth and Penner 1993). In \textit{M. sexta} ORNs, La\(^{3+}\) inhibited \approx 83\% of cyclic nucleotide-activated
currents. Low-La\(^{3+}\) concentrations, however, also potentiated
cyclic nucleotide-activated currents through prospective MsCNG
channels. Because the structure of prospective MsCNG channels
is unknown, and La\(^{3+}\) has not been used as an antagonist on CNG
channels before, the dose-dependent effects of La\(^{3+}\) on prospec-
tive MsCNG channels remain unclear. Nevertheless, a compara-
bale dose dependence of La\(^{3+}\) has been described for mouse
TRPC5 channels. At the extracellular mouth of the TRPC5 chan-
nel pore, lanthanides bind to specific glutamate residues, which
likely act as “gatekeepers” controlling cation entry (Jung et al.
2003). Remarkably, in \textit{M. sexta} ORNs, low-La\(^{3+}\) concentrations
appear to potentiate more cAMP- than cGMP-activated currents.
Thus cAMP- and cGMP-dependent prospective MsCNG channels
may differ in their channel pore composition.

Like La\(^{3+}\), transition metals such as Ni\(^{2+}\) and Zn\(^{2+}\) also
alter the sensitivity of CNG channels (Gordon and Zagotta

\textbf{FIG. 7.} Cyclic nucleotide-activated currents depend on the extracellular Cl\(^-\) concentration. \textit{I–V} relations of currents activated after application of 0.4 \(\mu\)M 8bcAMP (A) or
0.5 \(\mu\)M 8bcGMP (B) in 6Ca solution (I). Perfusion with low-Cl solution (2) significantly shifted the reversal potential of both cyclic nucleotide-activated currents to more negative values. Subsequent La\(^{3+}\) applica-
tion (3) inhibited the cyclic nucleotide-activated currents, but did not significantly change the mean reversal potential.

\textbf{FIG. 8.} Cyclic nucleotides activated currents in the presence of protein kinase inhibi-
tors. \textit{I–V} relations of currents activated after application of 0.5 \(\mu\)M 8bcAMP (A) and 0.5
\(\mu\)M 8bcGMP (B) in the presence of 1 \(\mu\)M staurosporine, or 1.5 \(\mu\)M 8bcAMP (C) and
1.5 \(\mu\)M 8bcGMP (D) in the presence of 10
\(\mu\)M H7. Subsequent La\(^{3+}\) application (2) inhibited the cyclic nucleotide-activated currents. C: in the presence of 10 \(\mu\)M H7, an inward current \(\textit{l}_{\text{Ca(cAMP)}}\) developed immedi-
ately after 8bcAMP application. The \(\textit{l}_{\text{Ca(cAMP)}}\) showed the characteristics of a
low-voltage-activated (LVA) Ca\(^{2+}\) current. While the \(\textit{l}_{\text{Ca(cAMP)}}\) (1) declined within sec-
onds to minutes, the cAMP-activated nonse-
selective cation current (2) developed.
Cyclic nucleotides induce an intracellular Ca\textsuperscript{2+} increase in vertebrate ORNs that subsequently leads to the activation of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} currents (Fringes et al. 2000). The secondary Cl\textsuperscript{-} efflux forms a large fraction of the current through vertebrate olfactory CNG channels. When Cl\textsuperscript{-} is replaced with larger anions, the corresponding current is eliminated (Kleene 1993). In \textit{M. sexta} ORNs, however, the replacement of Cl\textsuperscript{-} with acetate typically increased both cAMP- and cGMP-activated currents. Thus Cl\textsuperscript{-} seems to inhibit monovalent cation flux through prospective MsCNG channels. Since the replacement of Cl\textsuperscript{-} with acetate also significantly shifted the reversal potentials of the cyclic nucleotide-activated currents did not significantly change on La\textsuperscript{3+} application, the remaining La\textsuperscript{3+}-insensitive current could not be distinguished.

**Conclusions**

To our knowledge, there are only few reports on CNG channels in moths. In \textit{Heliothis virescens}, a cAMP- and hyperpolarization-activated CNG channel localized to antennal cells (Krieger et al. 1999). A clone from a cDNA library of \textit{Spodoptera littoralis} (P. Lucas and E. Jaquin-Joly, personal communication) showed high sequence similarity to invertebrate hyperpolarization-activated CNG channels (Gisselmann et al. 2003; Krieger et al. 1999; Quesneville et al. 2005). In addition, preliminary PCR studies suggested at least two CNG channel types in \textit{M. sexta}, among them one with sequence similarity to a subunit of the heterotetrameric CNG channels and another one with sequence similarity to hyperpolarization-activated CNG channels (M. Stengl and A. Nighorn, unpublished data).

Our patch-clamp results suggest that \textit{M. sexta} ORNs express at least one cAMP- and two cGMP-dependent prospective MsCNG channels. These prospective MsCNG channels differ in their Ca\textsuperscript{2+} dependence and Ca\textsuperscript{2+}/CaM-dependent inhibition. Therefore like vertebrate olfactory CNG channels, prospective MsCNG channels are probably composed of principal and modulatory subunits and may differ in their subunit composition. Besides the prospective MsCNG channels, \textit{M. sexta} ORNs express at least one cAMP-activated, protein kinase-dependent LVA Ca\textsuperscript{2+} current. Furthermore, \textit{M. sexta} ORNs express a delayed rectifier potassium channel of unknown molecular identity, which is inhibited by cyclic nucleotides (Stengl et al. 1992; Zufall et al. 1991). Thus a multiplicity of cyclic nucleotide-activated channels—nonselective cation, potassium, and calcium channels—are present in ORNs. Since both cAMP and cGMP activated ≥81% of ORNs, most ORNs appear to coexpress cAMP- and cGMP-dependent prospective MsCNG channels. The prospective MsCNG channels are thus likely to play a prominent role in the modulation of the olfactory transduction cascade. Current molecular cloning studies aim to identify the MsCNG channels that are involved in olfactory transduction.

**Acknowledgments**

We thank J. Benzler, C. Ellendt, S. Fastner, and M. Kern for insect rearing; P. Lucas for valuable discussions; and C. Wegener and M. Vömel for improvement of the manuscript.

**Grants**

This work was supported by Deutsche Forschungsgemeinschaft Grant STE 531/13-1,2 to M. Stengl.

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