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Dibattista M, Mazzatenta A, Grassi F, Tirindelli R, Menini A. Hyperpolarization-activated cyclic nucleotide-gated channels in mouse vomeronasal sensory neurons. J Neurophysiol 100: 576–586, 2008. First published May 28, 2008; doi:10.1152/jn.90263.2008. Hyperpolarization-activated currents (Ih) are present in several neurons of the central and peripheral nervous system. However, Ih in neurons of the vomeronasal organ (VNO) is not well characterized. We studied the properties of Ih in sensory neurons from acute slices of mouse VNO. In voltage-clamp studies, Ih was identified by the characteristic kinetics of activation, voltage dependence, and blockade by Cs+ or ZD-7288, two blockers of the Ih. Forskolin, an activator of adenylyl cyclase, shifted the activation curve for Ih to less negative potentials. A comparison of Ih properties in VNO neurons with those of heterologously expressed hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, together with RT-PCR experiments in VNO, indicate that Ih is caused by HCN2 and/or HCN4 subunits. In current-clamp recordings, blocking Ih with ZD-7288 induced a hyperpolarization of 5.1 mV, an increase in input resistance, a decrease in the sensitivity to elicitation actions in response to small current injections, and did not modify the frequency of action potentials elicited by a large current injection. It has been shown that in VNO neurons some pheromones induce a decrease in cAMP concentration, but the physiological role of cAMP is unknown. After application of blockers of adenylyl cyclase, we measured a hyperpolarization of 5.1 mV in 11 of 14 neurons, suggesting that basal levels of cAMP could modulate the resting potential. In conclusion, these results show that mouse VNO neurons express HCN2 and/or HCN4 subunits and that Ih contributes to setting the resting membrane potential and to increase excitability at stimulus threshold.

INTRODUCTION

Detection of most pheromonal cues requires the integrity of the vomeronasal organ of Jacobson (VNO), an accessory olfactory chemosensory organ that lies at the base of the nasal septum (Halpern and Martinez-Marcos 2003). The VNO epithelium is organized in multiple layers of bipolar sensory neurons, provided with dendrites and microvilli at the apical pole and axons at the basal side. The sensory axons contact the dendrites of mitral cells in the glomeruli of the accessory olfactory bulb and, from here, the pheromonal information is transmitted to regions of the brain dedicated to the neuroendocrine control (Brennan and Zuffal 2006; Zuffal and Leinders-Zuffal 2007). Vomeronasal sensory neurons have been divided into two classes based on their location in the sensory epithelium and on the type of receptors they express. Apical neurons are located closer to the luminal space and express V1R and the G protein Ga12, whereas basal neurons are located closer to the periphery of the sensory epithelium and express V2R and Ga13 (Berghard and Buck 1996; Dulac and Axel 1995; Jia and Halpern 1996; Ryba and Tirindelli 1997).

The nasal cavity of mammals also contains the main olfactory epithelium, mainly devoted to detect small volatile molecules, but also involved in responses to pheromones (Brennan and Zuffal 2006; Zuffal and Leinders-Zuffal 2007). Sensory neurons of both systems are bipolar neurons, and signal transduction occurs in specialized structures at their apical poles: cilia in olfactory and microvilli in vomeronasal neurons. The transduction mechanisms are different in the two systems. In olfactory sensory neurons, odorant receptor activation leads to cAMP production and to the opening of ciliary cyclic nucleotide gated (CNG) channels that, by triggering Ca influx, produce the subsequent activation of Ca-gated chloride currents that further contribute to membrane depolarization (Boccaccio and Menini 2007; Bradley et al. 2005; Kleene 1993; Kurahashi and Yau 1993; Lowe and Gold 1993; Pifferi et al. 2006). In contrast, membrane depolarization in vomeronasal sensory neurons on pheromone receptor stimulation, and G protein activation involves a diacylglycerol-activated cation channel, which depends in part on the transient receptor potential channel 2 (TRPC2), which triggers calcium and sodium influx (Kellihier et al. 2006; Liman et al. 1999; Lucas et al. 2003). Interestingly, VNO neurons highly express adenylyl cyclase ACII (Berghard and Buck 1996) and ACV1 (Rossler et al. 2000), as well as phosphodiesterase PDE1 and PDE4 (Cherry and Pho 2002), suggesting that, in VNO neurons, cAMP may play a physiological role. Indeed, it has been shown that some pheromones induce a reduction of the cAMP level in the garter snake (Luo et al. 1994) and in rodents (Luo et al. 1994; Rossler et al. 2000; Zhou and Moss 1997). However, whereas in olfactory neurons of the main olfactory system cAMP is the primary second messenger of the transduction system and its concentration rapidly increases on odorant stimulation, in VNO neurons, the decrease in cAMP occurs with a longer delay with respect to pheromone stimulation (Rossler et al. 2000). Although it has been well established that cAMP is not a primary second messenger in VNO neurons, its physiological role in the VNO is still unknown. Possible targets for modulatory actions by cAMP include the hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels. HCN
channels are members of the voltage-gated ion channel superfamily and contain a cyclic-nucleotide binding domain in their carboxy terminus region (Zagotta et al. 2003). To date, a family of four genes (HCN1-4) has been identified (Ludwig et al. 1998; Santoro et al. 1998). HCN isoforms differ for amino acid sequence, tissue distribution, and for their capacity to form functional heteromeric channels. Moreover, when heterologously expressed, HCNs show distinct cAMP sensitivity and gating properties (Craven and Zagotta 2006; Guth and Dietze 1995; Ingram and Williams 1996).

HCN channels have been shown to be responsible for the hyperpolarization-activated inward current ($I_h$) (Brown et al. 1979). This type of current, originally termed “funny” or “queer” because of its unusual properties of being activated by hyperpolarization and being inward, was first discovered in cardiac sinoatrial cells (Brown et al. 1979) and is involved in the physiology of several neurons of the central and peripheral nervous system (Maccarelli et al. 1993; Pape 1996). Trotier and colleagues first showed that frog and rat VNO neurons express $I_h$ and that this current concurs to the setting of the resting potential (Trotier and Dovign 1996; Trotier et al. 1993, 1998). More recently, in mouse VNO neurons, Ukhanov et al. (2007) have recorded a voltage “sag” induced by hyperpolarizing current steps, which is typical of $I_h$ currents. However, most properties of $I_h$ in mouse VNO neurons have not been characterized, including modulation by cAMP.

In this study, we measured the biophysical properties and investigated the physiological role, including modulation by cAMP, of $I_h$ in mouse vomeronasal sensory neurons to further contribute to the understanding of the molecular mechanisms of pheromonal signaling.

**METHODS**

**Animals**

All experiments were performed on adult C57Black J/6, 129/Sv, and FVB mice (P60–P90) in accordance to both international guidelines and Italian Guidelines for the Use of Laboratory Animals (Decreto Legislativo 27/01/1992, no. 116).

**Preparation of isolated vomeronasal sensory neurons**

Mice were decapitated after anesthesia by inhalation of CO$_2$, and the VNO capsule was removed. For experiments with isolated neurons, vomeronasal sensory neurons were dissociated from the VNO with a standard enzymatic–mechanical dissociation protocol, as previously described (Dean et al. 2004). The extracellular mammalian Ringer solution contained (in mM) 140 NaCl; 5 KCl; 1 CaCl$_2$; 1 MgSO$_4$; 10 HEPES hemisodium; 1 sodium pyruvate; and 10 glucose; pH 7.4. The high K$^+$ solution contained (in mM) 120 NaCl; 25 KCl; 1 CaCl$_2$; 1 MgCl$_2$; 10 HEPES hemisodium; 1 sodium pyruvate; and 10 glucose; pH 7.4.

**Preparation of and acute slices of mouse VNO**

Acute coronal slices of the VNO were prepared as described by Shimazaki et al. (2006). The VNO was dissected in ice-cold saline solution containing (in mM) 120 NaCl, 25 NaHCO$_3$, 5 KCl, 5 HEPES, 1 MgSO$_4$, 1 CaCl$_2$, and 10 glucose (osmolarity adjusted to 300 mOsm, pH 7.3), oxygenated with 95% O$_2$–5% CO$_2$. Each half of the VNO was embedded in 0.5% low-grade agar (A7002, Sigma) prepared with 9.9% saline solution. Coronal slices of 250 μm were cut with a vibratome (Vibratome 1000 Plus Sectioning System). Slices were transferred to a recording chamber continuously perfused with oxygenated saline solution and were viewed with an upright microscope (Olympus BX51WI) by infrared differential contrast optics with water immersion ×10 or ×40 objectives. The VNO slices maintained their cross-sectional structure, and many individual neurons could be distinguished by their morphology. Whole cell experiments were obtained by patching the soma of neurons presumably belonging to the basal layer, although we could not determine with certainty if the neurons were located in the basal or in the apical layer. Once in the whole cell configuration, it was possible to make continuous recordings for ≤2 h.

In voltage-clamp experiments, the high K$^+$ solution contained (in mM) 100 NaCl, 25 NaHCO$_3$, 25 KCl, 5 HEPES, 1 MgSO$_4$, 1 CaCl$_2$, and 10 glucose (osmolarity adjusted to 300 mOsm, pH 7.3).

**Electrophysiology**

Whole cell recordings from individual neurons were obtained with the Axopatch 1D patch-clamp amplifier (Axon Instruments) in the voltage- and current-clamp mode. Patch pipettes were made using borosilicate microcapillary glass (WPI, Sarasota, FL) and pulled with a two-stage puller (model PP-830, Narishige, Tokyo, Japan). The intracellular pipette solution contained (in mM) 145 KCl; 4 MgCl$_2$; 10 HEPES hemisodium; 0.5 EGTA; 1 ATP; and 0.1 GTP; pH 7.4. In some experiments, 1 mM BaCl$_2$, 2 or 5 mM CsCl, 0.1 mM ZD-7288 (4-(N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride (Tocris Bioscience), 10 μM forskolin, or 50 μM MDL-12330A together with 100 μM SQ 22536 were added to the extracellular solutions. A multibarrel perfusion system was used to exchange extracellular solutions. Pipette resistances were 3–10 MΩ when filled with the intracellular solution. Currents were low-pass filtered at 5 kHz (Kemo, Beckenham, Kent, UK) and digitized at 10–20 kHz with an A/D interface (Digidata 1322A, Axon Instruments) and PClamp 9.2 software (Axon Instruments). Experiments were performed at room temperature (21–23°C). All chemicals were from Sigma unless otherwise stated.

**Data analysis**

Data analysis and figures were performed using Clampfit 9.2 and Igor software (WaveMetrics, Lake Oswego, OR). Data are given as mean ± SE and the total number of neurons (n). Statistical significance was tested with a Student’s t-test. P < 0.05 was considered statistically significant.

**RNA extraction**

Total RNAs were obtained from VNO, heart, and brain using the RNAzol B extraction method (Biotech Italia, Roma, Italy) according to the manufacturer’s recommendations. The quality and amount of the RNA was evaluated by gel electrophoresis on agarose gel.

**cDNA synthesis**

cDNA was synthesized by reverse transcription (60 min at 55°C) from total RNA (4 μg) in a reaction mixture containing 2.5 μM oligo (dT)$_{20}$ primer (Invitrogen, Milan, Italy), 4 μl 5x First Strand Buffer, 1 μl of a dNTP (each at a 10 mM concentration), 1 μl 0.1 M DTT, 0.5 μl RNAGuard RNase inhibitor (25,600 U/ml; Amershams Pharmacia Biotech), and 1 μl of Superscript III RT (200 U/μl; Invitrogen). The reaction was inactivated by heating at 70°C for 15 min. The resulting cDNA was purified on Bio-Spin 6 columns (BioRad).

**PCR conditions**

Reaction mixtures were assembled with the following components: 2 μl cDNA, 5 μl reaction buffer [160 mM (NH$_4$)$_2$SO$_4$, 670 mM
Tris-HCl pH 8.8, 0.1% Tween 20), 1.5 μl MgCl₂, 50 mM, 1.5 μl primers (2.5 pmol/μl), 1 μl of 10 mM dNTP, 0.3 μl Taq DNA polymerase (5 U/μl; Euroclone, Milan, Italy), and water to 50 μl. The annealing temperature was 53°C for 40 cycles. The sequence of the primers used were as follows: HCN1_FW, 5’-AGTGTGATTTGACCGACTCGA-3’ and HCN1_RE, 5’-GCTGCTGCTGCTGCTGC-3’ (for mouse HCN1 525 bp; NCBI accession no. NM_010408); HCN2_FW, 5’-GAAGATGTATCCTCCACAGCA-3’ and HCN2_RE, 5’-CTGGCAAGCTCTGCCTG-3’ (for mouse HCN2 391 bp; NCBI accession no. NM_008226); HCN3_FW, 5’-GAAGATGTATCACCACAGCA-3’ and HCN3_RE, 5’-AGCCAGAATGIGCCGGCAGCCC-3’ (for mouse HCN3 773 bp; NCBI accession no NM_008227); HCN4_FW, 5’-GGATATCCCATGAGGGAG-3’ and HCN4_RE, 5’-GCCAGAGGGAATCACAATCCA-3’ (for mouse HCN4 513 bp; NCBI accession no. NM_001081192).

All primers were designed across the exon–intron boundaries. The size of the PCR products was determined by agarose gel. DNA was gel-extracted using QIAquick gel extraction kit (Quiagen, Hilden, Germany) following the manufacturer’s instructions. The DNA fragments obtained were cloned into pGEM-T Easy Vector Systems (Promega, Madison, WI) and sequenced.

Immunohistochemistry

For immunohistochemistry, 2-mo-old FVB mice were used. Mice were deeply anesthetized with pentobarbital and transcardially perfused with an ice-cold fixative containing 2% paraformaldehyde and 15% saturated picric acid in PBS buffer. The VNO was immediately dissected, postfixed for 2 h, and cryo-protected overnight at 4°C in 30% sucrose. Tissues were included in OCT embedding solution (CellPath, Newtown, UK) and frozen. Cryostat cut sections (16 μm) were dried and blocked with a solution containing 1% albumin and 0.3% Triton X-100 in PBS for 30 min before incubation with the primary antibody. Anti- HCN2 and anti-HCN4 antibodies (Alomone Laboratories, Jerusalem, Israel) were used at a dilution of 1:100. After antibody incubation, sections were rinsed with PBS and developed with a biotinylated secondary antibody (Biotinylated Anti-Rabbit IgG, Vector Laboratories, Burlingame, VT) and the TSA biotin system (PerkinElmer, Boston, MA) according to the manufacturer’s instructions. For preabsorption controls, 1 μg of each antibody was incubated with 5 μg of the polypeptide against which the antibody was raised. Fluorescent images were obtained using a Zeiss fluorescent microscope.

RESULTS

Inward currents activated by hyperpolarization and their pharmacological characterization

Currents activated by hyperpolarization in isolated mouse vomeronasal sensory neurons were measured in mammalian Ringer with 5 mM K⁺, and in high K⁺ solutions, containing 25 mM K⁺. Examples of currents of typical amplitudes are shown in Fig. 1A. Slowly activating inward currents were evoked at membrane potentials more negative than −100 mV. When the extracellular K⁺ concentration was increased, the current amplitude increased without a shift in the voltage range of activation, as shown in Fig. 1B. Both amplitude and time constant of activation of the inward current were voltage dependent (see also Fig. 2).

We examined the effects of Ba²⁺, Cs⁺, and ZD-7288 on the inward currents activated by hyperpolarization to distinguish between the contributions of Iₖ and inward rectifier K⁺ channels (Kir) currents. Indeed, 1 mM Ba²⁺ is a well-known blocker of Kir currents (Nichols and Lopatin 1997), and it does not affect Iₖ (at concentrations <2 mM); Cs⁺ is a blocker of both currents (DiFrancesco 1995), and ZD-7288 is a heart rate limiting agent, used as a specific blocker of Iₖ in different cell types (BoSmith et al. 1993; Gasparini and DiFrancesco 1997; Satoh and Yamada 2002).

Figure 1, C, D, and H, shows that the inward current was not significantly affected by the addition of 1 mM BaCl₂ to the
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Figure 2. Kinetics of $I_h$ current activation. A: current traces in high K$^+$ solution and (B) after the addition of 5 mM CsCl. Currents were elicited by voltage steps of variable durations in 20-mV increments from −100 to −190 mV from a holding potential of −60 mV. C: traces obtained by subtracting the currents blocked by Cs$^+$ in B from the traces at the corresponding voltages in A. The activation time constant, $\tau$, at each voltage was obtained from the fit (continuous lines) of current responses to a single exponential function, $I(t) = I_s(1 - e^{-t/\tau})$, where $I_s$ is the current amplitude at the steady state. D: voltage dependence of the average $\tau$ values for currents recorded in high K$^+$ ($n = 5$; filled circles) and in Ringer ($n = 3$; open squares).

bathing solution, whereas it was reversibly blocked by 2 mM CsCl. The addition of the specific $I_h$ current blocker ZD-7288 at 100 $\mu$M blocked the inward current (Fig. 1, E, F, and H) with a partial recovery of the current after blocker removal. Figure 1H shows the average current reduction measured at −150 mV in high K$^+$ solution from several cells in the presence of Ba$^{2+}$, Cs$^+$, or ZD-7288. The absence of a significant blockage by Ba$^{2+}$ and the presence of a blockage by Cs$^+$ and by ZD-7288 indicate that the measured inward currents are $I_h$ currents (DiFrancesco 1995; Pape 1996). In subsequent experiments, we often chose Cs$^+$ as an $I_h$ blocker because its blockage was fast and completely reversible compared with the slow and only partially reversible blockage by ZD-7288.

Activation kinetics

Recordings in Fig. 1 showed that the activation of inward currents was slow and was still increasing at the end of the 200-ms voltage pulse. However, in recordings from isolated neurons, it was often difficult to significantly increase the duration of the hyperpolarizing step at voltages more negative than −110 mV without losing the tight seal. Recordings obtained from individual neurons in slices of the VNO have been shown to be more stable (Shimazaki et al. 2006; Ukhanov et al. 2007) and longer lasting, and therefore we performed most of the subsequent experiments in VNO slices. Moreover, since current amplitudes measured in Ringer solution were very small (Fig. 1A), in most voltage-clamp recordings, a high K$^+$ solution was used to increase $I_h$ current amplitudes.

The activation kinetics of $I_h$ were calculated by fitting the current traces at each hyperpolarized potential to a single exponential function, as shown in Fig. 2. $I_h$ currents were considered as the Cs$^+$-sensitive components (Fig. 2C) measured by subtracting the current traces recorded in the presence of Cs$^+$ (Fig. 2B) from the corresponding traces in the absence of Cs$^+$ (Fig. 2A). The activation time constant ($\tau$) was voltage-dependent and was increasingly smaller at more hyperpolarized potentials. Fits of a single exponential function are shown in Fig. 2C, and the means are shown in Fig. 2D. The comparison between $\tau$ values obtained in Ringer and high external K$^+$ at the same potentials shows that a change in K$^+$ concentration did not affect the kinetics of $I_h$ activation (Fig. 2D). At −150 mV, the mean $\tau$ for $I_h$ was 314 ± 35 ms and was reduced to 124 ± 10 ms at −190 mV ($n = 7$), whereas at −100 mV, $\tau$ increased to 2.7 ± 0.4 s ($n = 5$).

Voltage dependence of $I_h$ activation

The voltage dependence of current activation was determined by tail current activation curves. Currents were activated from the holding potential of −60 mV by a first hyperpolarizing prepulse potential varying between −100 and −180 mV and were fully activated by a voltage step at −180 mV (Fig. 3A). Since pulses of longer durations are necessary to reach current steady state at the less hyperpolarized potentials, as shown by the voltage dependence of $\tau$ in Fig. 2, we used pulses of different length to minimize the time the neuron was held at the most negative potentials. The amplitude of the tail current measured after stepping the potential to −180 mV was plotted as a function of the hyperpolarizing prepulse used to activate the current. The voltage dependence of activation was determined by fitting the amplitude of tail currents to a Boltzmann function

$$I = I_{\text{max}}/[1 + \exp((V - V_{\text{half}})/k_s)]$$

where $I_{\text{max}}$ is the maximal current, $V$ the prepulse potential, $V_{\text{half}}$ the half-activation potential, and $k_s$ is the slope factor. Figure 3B shows tail currents plotted versus the prepulse potential from the experiment shown in Fig. 3A. The estimated values were −120 mV for $V_{\text{half}}$ and 15 mV for $k_s$. 

FIG. 3. $I_h$ current activation curves. A: currents in high K$^+$ solution were elicited by the shown voltage protocol and the currents blocked by Cs$^+$ were subtracted as shown in Fig. 2. B: tail current amplitudes were measured at −180 mV following return from a series of prepulse potentials. The best fit of the activation curve to the Boltzmann Eq. 1 gave the following values $V_{\text{half}} = −120$ mV, $k_s = 15$ mV.

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Average values were $-118 \pm 4$ mV for $V_{\text{half}}$ and $17 \pm 2$ mV ($n = 4$).

In two neurons, we also estimated the reversal potential of $I_h$. Currents were activated from a holding potential of $-60$ mV with a first prepulse potential step at $-180$ mV, followed by step pulses varying between $-140$ and $-90$ mV. The reversal potential value, extrapolated by fitting data with a straight line, was about $-32$ mV (data not shown). The permeability ratio between Na$^+$ and K$^+$ ions calculated from the Goldman-Hodgkin-Katz equation gave a value of 0.11, consistent with a mixed permeability of both K$^+$ and Na$^+$ ions as expected for $I_h$.

Modulation of $I_h$ by forskolin

To study whether $I_h$ in VNO neurons is modulated by cyclic nucleotides, we added 10 $\mu$M forskolin, a potent nontspecific adenylyl cyclase activator, to the extracellular high K$^+$ solution, to increase cAMP intracellular concentration (El Kholy et al. 2007; Michels et al. 2005; Okamoto et al. 1996). Figure 4A shows recordings from the same neuron before and after addition of forskolin. At $-120$ mV, the average $\tau$ was $671 \pm 68$ ms and decreased to $516 \pm 10$ ms after the addition of forskolin ($n = 4$; $P < 0.05$; Fig. 4B), whereas the average normalized $I_h$ amplitude at the steady state increased to $1.29 \pm 0.05$ after addition of forskolin ($n = 4$; $P < 0.05$; Fig. 4C).

The normalized activation curve in the presence of forskolin was measured and compared with that in control in the same neurons (Fig. 5). The average $V_{\text{half}}$ shifted from $-118 \pm 4$ mV in control to $-109 \pm 5$ mV after addition of forskolin ($n = 4$; $P < 0.05$; Fig. 5C), whereas the average slope $k_1$ did not significantly change from $17 \pm 2$ to $15 \pm 2$ mV ($n = 4$; $P > 0.05$; Fig. 5D). The average value of the maximal current changed from $-84 \pm 12$ to $-103 \pm 14$ pA ($n = 4$; $P < 0.05$) following addition of forskolin.

Therefore the increase of cAMP induced by forskolin seems to modulate $I_h$ in mouse vomeronasal sensory neurons by inducing a shift of the voltage dependence of activation to more positive potentials of $\sim 9$ mV and by increasing the absolute value of the maximal current of $\sim 123\%$.

Blocking $I_h$ in current-clamp recordings: resting potential and input resistance

To study the functional role of $I_h$ in physiological conditions, we obtained current-clamp recordings in standard Ringer solution. We used the whole cell current-clamp technique to measure the membrane potential of sensory neurons in VNO slices at rest or in response to hyperpolarizing current steps (Fig. 6). When hyperpolarizing current steps were applied, the membrane potential showed an initial hyperpolarization followed by a slow depolarization, known as depolarizing sag (Fig. 6A). The addition of CsCl, or of ZD-7288, abolished the depolarizing sag (Fig. 6, A and C). On termination of the hyperpolarizing current steps, rebound action potentials were measured both in the absence and in the presence of CsCl or ZD-7288.

Resting membrane potentials in Ringer solution, referred to as 0-pA current injection, varied between $-88$ and $-58$ mV,
with an average value of $-67 \pm 1$ mV ($n = 38$). Bath application of Cs caused a hyperpolarization of the membrane potential of $6.5 \pm 1.7$ mV ($-60.6 \pm 2.3$ to $-67.1 \pm 2.6$ mV; $n = 4$; $P < 0.05$), indicating that Cs inhibited an inward current at the resting potential (Fig. 6, A and B). Moreover, the input resistance increased from $4.1 \pm 0.7$ to $10.9 \pm 0.9$ GΩ ($n = 4$; $P < 0.001$) after addition of CsCl (Fig. 6B).

We obtained similar results with the specific Ih blocker ZD-7288. The average resting membrane potential hyperpolarized of $5.1 \pm 0.9$ mV (from $-65.4 \pm 2.6$ to $-70.5 \pm 2.3$ mV; $n = 10$; $P < 0.001$), and the input resistance increased from $4.1 \pm 0.8$ to $5.1 \pm 0.9$ GΩ ($n = 3$; $P < 0.05$) after addition of ZD-7288 (Fig. 6C).

These results show that Ih is active at the resting membrane potential in mice VNO neurons.

Ih and excitability of VNO neurons

We first measured the excitability of VNO neurons by measuring the threshold stimulus current necessary to elicit action potentials at different membrane potentials. Figure 7 shows responses to a series of small current pulses of 2-s duration increasing in steps of 0.2 pA from a VNO neuron at different membrane potentials. The resting potential was near $-60$ mV, and the first trace on the left shows that a current injection of only 0.2 pA was sufficient to elicit action potentials. However, when a constant current injection of $-1.9$ pA brought the membrane potential of the same neuron to about $-80$ mV, a depolarizing step of 2 pA was necessary to elicit one action potential. Similar results were obtained in three other neurons.

In another set of experiments we studied whether Ih modifies the excitability of VNO neurons at threshold. The spike threshold in response to a series of small current injections was measured in control conditions and in the presence of ZD-7288 (Fig. 8). The resting potential of this neuron was near $-73$ mV and became about $-78$ mV after addition of ZD-7288. A step of 1.0 pA elicited one action potential in control conditions, whereas after Ih blockage, a higher current step of 1.6 pA was necessary to cause an action potential. Further current injections progressively increased firing frequency. Similar results were measured in two other neurons.

We next studied whether Ih blockage modified the frequency of action potentials in response to a 5-pA current step. We compared current-clamp recordings in response to a depolarizing current step in both control solution and after blocking Ih with ZD-7288 in the same neurons (Fig. 9). As shown in Fig. 6, the addition of the blocker produced a hyperpolarization of the resting potential at 0-pA current injection. Depolarizing current steps of 5-pA amplitude and 3-s duration elicited repetitive action potentials (Fig. 9, A and B). The average frequency was $4.5 \pm 0.4$ Hz in control and $4.4 \pm 0.3$ Hz after addition of ZD-7288 ($n = 6$; $P > 0.05$; Fig. 9C). Therefore blocking Ih currents with ZD-7288 does not modify the frequency of action potentials induced by depolarizing current steps in VNO neurons.

Taken together these results indicate that Ih modulates the excitability of VNO neurons by lowering the threshold to stimulus current for action potential generation, whereas it does not modify the frequency of action potentials induced by a large depolarizing current step.

Modulation by cAMP

Changes in intracellular concentrations of cAMP modulate HCN channels, and we show in Fig. 5 that the addition of 10 μM forskolin caused a shift in the activation curve of Ih of 9 mV toward less negative potentials. To study whether changes in intracellular cAMP produce changes in more physiological conditions, we measured the effects of the adenyl cyclase inhibitors SQ 22536 and MDL-12330A in current-clamp recordings. Figure 10 shows an example of the effect of 100 μM SQ 22536 together with 50 μM MDL-12330A on the resting potential and on the input resistance of a VNO neuron. In this neuron, the resting potential hyperpolarized from $-60$ to $-65$ mV. Similar effects were observed in 11 of 14 neurons tested. Bath application of SQ 22536 and MDL-12330A caused a...
hyperpolarization of the membrane potential of $5.1 \pm 0.9$ mV (from $-63.4 \pm 2.3$ to $-68.6 \pm 2.5$ mV; $n = 11; P \ll 0.05$), indicating a reduction of the inward current active at the resting potential (Fig. 10B). The average input resistance increased from $4.2 \pm 0.4$ to $4.9 \pm 0.6$ GΩ in the presence of SQ 22536 and MDL12,330A ($n = 6; P < 0.05$). In five neurons, we could also test the effect of adding ZD-7288 (Fig. 10A), and we measured a further average hyperpolarization from $-69.8 \pm 4.3$ (in SQ 22536 and MDL-12330A) to $-72.8 \pm 4.7$ mV (after addition of ZD-7288; $n = 5; P < 0.05$; Fig. 10C).

**Identification of HCN subunits in the VNO**

To examine the putative subunit composition of $I_h$ channels, we analyzed the expression of the known HCN isoforms in RT-PCR experiments on VNO tissue. Specific primers for each of the four HCN isoforms showed that only HCN2 and HCN4 are expressed in the VNO (Fig. 11). As a control for the primer and cDNA quality, we also amplified all HCN isoforms cDNA from brain and heart. HCN1-4–specific primers amplified cDNA PCR products of the predicted size (HCN1, 525 bp; HCN2, 391 bp; HCN3, 409 bp; HCN4, 513 bp; Fig. 11). All PCR fragments were excised, purified, and sequenced to confirm the identity of the different HCN isoforms. Furthermore, a semiquantitative analysis based on the amplification of β-actin from the whole VNO cDNA as a reference indicates that HCN2 and HCN4 isoforms are expressed at a very low level in the VNO. This observation is also supported by the lack of reproducible results when VNO sections were assayed for immunoreactivity and RNA expression by in situ hybridization with antibodies and probes against the different HCN isoforms (data not shown).

**DISCUSSION**

In this study, we characterized some properties of $I_h$ in mouse vomeronasal neurons, such as the modulation by cAMP, the contribution to the resting membrane potential, and the role...
in excitability. Moreover, we identified the HCN isoforms that are likely to be responsible for the onset of $I_h$ in the VNO.

Previous reports (Liman and Corey 1996; Trotier and Doving 1996; Trotier et al. 1993, 1998) showed evidence of $I_h$ in vomeronasal sensory neurons and for its role in setting the resting membrane potential, thus balancing the hyperpolarization-induced by the Na$^+$,K$^+$-ATPase (Trotier and Doving 1996). In a recent study Ukhano et al. (2007) characterized the electrophysiological properties of basal and apical mouse VNO neurons using transgenic mice in which VNO neurons express green fluorescent protein (GFP) under the control of either the V1rb2 or V2r1b receptor gene and found that injection of hyperpolarizing currents showed the presence of a hyperpolarization-activated “sag,” typical of $I_h$ activation, in both neuron types.

Here, we pharmacologically identified $I_h$ in mouse VNO neurons as being insensitive to Ba$^{2+}$ and blocked by ZD-7288, a selective antagonist of $I_h$. Furthermore, we measured activation curves and estimated, from fits to the Boltzmann equation, a selective antagonist of $I_h$ and activated from the Boltzmann equation, an average $V_{1/2}$ of $-118$ mV with $k_a = 17$ mV. To the best of our knowledge, there are no previous measurements of activation curves for $I_h$ in mouse VNO neurons. Instead, in cultured and dissociated rat olfactory sensory neurons, it has been estimated that $V_{1/2}$ values range between $-142$ (with $k_a = 10$ mV), and $-117$ mV (with $k_a = 5.5-10$ mV) (Lynch and Barry 1991a; Vargas and Lucero 1999). Therefore activation curves for both vomeronasal and olfactory sensory neurons seem to be quite similar.

Other similar features of the $I_h$ currents in chemosensory neurons of the two olfactory subsystems include the modulation of $I_h$ by cAMP (Vargas and Lucero 1999, 2002) and the kinetics of activation of these currents (Lynch and Barry 1991a).

Indeed, Vargas and Lucero (1999) showed, in the rat, that the addition of 1 mM cAMP to the intracellular solution generated a 28-mV depolarizing shift (from $-118$ to $-90$ mV) in the activation curve of $I_h$ without a significant change in the slope factor. In VNO neurons, we also measured a depolarizing shift following the application of the adenylyl cyclase activator forskolin, with a shift of 9 mV (from $-118$ to $-109$ mV) and no variation in the slope factor.

In our hands, the kinetics of $I_h$ activation in VNO neurons show a time constant $\tau$ of, respectively, 314 ms at $-150$ mV and 568 ms at $-120$ mV; conversely, slightly contrasting $\tau$ values were reported for the rat olfactory sensory neurons (275 ms at $-153$ mV and 1–2 s at $-120$ mV) (Lynch and Barry 1991a), thus suggesting that the two types of chemosensory neurons have similar but not identical slow activation kinetics.

What are the HCN functional isoforms in VNO neurons? The comparison between our data with those reported from recombinant mouse HCN channels expressed in heterologous systems (Ludwig et al. 1998; Santoro et al. 1998, 2000) prompts us to envisage an involvement of the HCN2 and HCN4 isoforms in the onset of $I_h$ in VNO neurons. For example, the activation kinetics that we have determined for $I_h$ are comparable to that observed in cultured cells expressing recombinant HCN2, HCN3, and HCN4, but not HCN1. Moreover, HCN2 and HCN4 are strongly modulated by cAMP, whereas HCN1 shows a minimal response to cAMP, and HCN3 is not modulated by cAMP (Ludwig et al. 1998; Moosmang et al. 1999; Santoro and Tibbs 1999; Santoro et al. 1998; Seifert et al. 1999; Steiber et al. 2005). Finally, our RT-PCR experiments with HCN isoform–specific primers highlight the presence of both HCN2 and HCN4 isoforms, whereas they failed to detect either HCN1 or HCN3 in the VNO.
the activation curve (Fig. 5) and estimated a value of 4 pA in current-clamp mode. The resting potential in Ringer was −60 mV and hyperpolarized to −65 mV after application of SQ 22536 and MDL-12330A. The average resting potential moved toward more negative values (n = 11; P < 0.05). C: in 5 of the 11 neurons, ZD-7288 was applied at the end of the experiment and the average resting potential further hyperpolarized (n = 5; P < 0.05).

**Physiological role: resting potential and VNO neuron excitability**

Our voltage-clamp experiments clearly show the presence of $I_h$ in mouse VNO neurons and show that $I_h$ properties are modulated by cAMP. We calculated the amplitude of $I_h$, at the average resting value of −67 mV, from the Boltzmann fit to the activation curve (Fig. 5) and estimated a value of 4 pA in high K+ solution. By taking into account that the $I_h$ amplitude in Ringer is −0.6 of that in high K+ (Fig. 1), the estimated current amplitude in Ringer solution must be reduced to 2.4 pA. Since VNO neurons have a high-input resistance of ~4 GΩ, a 2.4-pA increase in current will result in a depolarization of 9.6 mV of the membrane potential. Therefore even small $I_h$ amplitude can significantly affect their resting potential. It is worth mentioning here that the real resting membrane potential of VNO neurons is likely to be more negative than the observed value. As previously discussed by several authors (Lynch and Barry 1991a,b; Schild 1989; Schild and Restrepo 1998; Trotier and Doving 1996), the underestimation of the resting potential is a technical problem that occurs in cells that have a small size and a high-input resistance, as for vomeronasal and olfactory sensory neurons. Indeed, in frog vomeronasal sensory neurons, Trotier and Doving (1996) have shown that increasing the seal resistance between the cell membrane and the patch pipette modified the estimation of the resting potential from −61 to −88 mV. By a different method, Lynch and Barry (1991a,b) have calculated a resting membrane potential more negative than −90 mV in rat olfactory sensory neurons.

In current-clamp recordings, we showed that blocking $I_h$ with ZD-7288 caused a hyperpolarizing shift of the resting membrane potential of ~5 mV and that the input resistance increased from 4 to 5 GΩ. This entails that $I_h$ is active at the resting potential and contributes to setting its value in mouse VNO neurons.

What is the influence of $I_h$ on firing properties of VNO neurons? The $I_h$ properties would, in principle, influence the neuron excitability in two opposing ways. On one side, $I_h$ tonic activation depolarizes the resting potential and therefore could increase the probability of firing in response to a given positive current step. Conversely, the decrease in the input resistance caused by $I_h$ activation will produce a smaller voltage change in response to a given current step. Therefore if $I_h$ is reduced or blocked, a smaller stimulus could produce a larger depolarization. Which one of the two effects is predominant cannot be generally predicted, because influences may result from the different HCN isoforms expressed by a given cell or from the interplay with other currents than $I_h$ (Robinson and Siegelbaum 2003; Santoro and Baram 2003). In our experiments, a decrease of $I_h$ modified the current threshold for action potential generation by increasing the amplitude of the current step necessary to elicit an action potential. Instead, the frequency of action potentials elicited by a relatively large current step (~4.5 Hz at 5-pA current injection, in agreement with previous measurements; Shimazaki et al. 2006; Ukhanov et al. 2007) did not significantly change after blockage of $I_h$ with ZD-7288.

Thus in VNO neurons, $I_h$ seems to increase the neuron excitability at low stimulus currents without influencing the neuronal firing frequency at quite large depolarizing current steps.

$I_h$ as a candidate target for cAMP functional role in the VNO

The role of cAMP in pheromonal communication remains at present elusive in VNO neurons, although some components of...
the cAMP-signaling cascade as ACII, ACVI, PDE1, and PDE4 isoforms are abundantly expressed (Berghard and Buck 1996; Cherry and Pho 2002; Rossler et al. 2000). For example, in the rat VNO, urinary components have been reported to induce a decrease in cAMP concentration (Zhou and Moss 1997) as a consequence of the preceding activation of the phosphodiesterase pathway (Rossler et al. 2000). Thus cAMP, even if not the primary messenger of the transduction process in VNO neurons, might be responsible for at least some features of the pheromonal communication.

Here, we show that I_h is modulated by cAMP in mouse VNO neurons. Indeed, our voltage-clamp experiments indicate that the rise of the intracellular cAMP concentration, through the application of the adenyl cyclase inhibitors, SQ 22536 and MDL-12330A, in current-clamp conditions that resulted in a hyperpolarization of the resting membrane potential. Thus it is plausible that, if there are changes in cAMP levels in VNO neurons induced either on pheromonal stimulation (Rossler et al. 2000; Zhou and Moss 1997) or by other physiological mechanisms, they may influence the I_h amplitude and the excitability of VNO neurons in response to small stimuli.

In the VNO, it has been shown that apical and basal neurons possess some different electrophysiological properties (Ukhanov et al. 2007). Future experiments using mice genetically modified to allow the visual identification of apical and basal neurons should also study whether I_h properties are different in the two types of neurons.

In conclusion, these results represent the first systematic study on I_h currents in mouse VNO neurons. They show that 1) I_h contributes to setting the membrane resting potential and increases the sensitivity to elicit action potentials in response to small current injections, 2) I_h is positively modulated by cAMP, and 3) I_h is specifically generated by the expression of HCN isoforms in the mouse VNO. This introduces the possibility of exploiting the mutant mouse strains for the HCN2 or HCN4 gene to acquire relevant information about the physiological role of I_h and cAMP in pheromonal signaling (Herrmann et al. 2007; Ludwig et al. 2003; Stieber et al. 2003).

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