Patch-Clamp Analysis of Gene-Targeted Vomeronasal Neurons Expressing a Defined V1r or V2r Receptor: Ionic Mechanisms Underlying Persistent Firing

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Ukhanov K, Leinders-Zufall T, Zufall F. Patch-clamp analysis of gene-targeted vomeronasal neurons expressing a defined V1r or V2r receptor: ionic mechanisms underlying persistent firing. J Neurophysiol 98: 2357–2369, 2007. Sensory neurons in the mouse vomeronasal organ consist of two major groups, apical and basal, that project to different brain regions, express unique sets of receptors, and serve distinct functions. Electrical properties of these two subpopulations, however, have not been systematically characterized. V1rb2-tau-GFP and V2r1b-tau-GFP tagged vomeronasal sensory neurons (VSNs) were selected as prototypical apical or basal VSNs, respectively, and their biophysical properties were analyzed in acute slices that minimized cell damage. Basal V2r1b-expressing VSNs had voltage-gated conductances, and especially Na+ (Nav) and Ca2+ (Cav) currents, that were substantially larger than those observed in apical V1rb2 VSNs, although the resting membrane potential, input resistance, and membrane capacitance were similar in both cell types. Of several types of Cav currents, T-type and L-type Cav currents contributed to action potential firing, and both currents alone were capable of generating oscillatory Ca2+ spikes. The L-type Cav current was uniquely coupled to a BK large-conductance K+ current, and interplay between these channels played a critical role in repolarizing spikes and maintaining persistent firing in VSNs. Larger Nav and Cav conductances, along with a more positive inactivation voltage of the Nav current in the V2r1b VSNs, contributed to the larger spike amplitude and higher spike frequency induced by depolarizing current in these cells compared with V1rb2 VSNs. Basal GFP-negative VSNs and V2r1b VSNs responded to prolonged depolarization with persistent, but adapting discharge that could be relevant in sensory adaptation. Collectively, these results suggest a novel mechanism for regulating and encoding neuronal activity in the accessory olfactory system.

INTRODUCTION

The vomeronasal system has long been a focus of study in mammalian sensory physiology. In rodents, the vomeronasal organ (VNO) is responsible for detection and transmission of a wide variety of pheromonal cues that are critically involved in social and reproductive behavior (Brennan and Keverne 2004; Brennan and Zufall 2006; Dulac and Torello 2003; Spehr et al. 2006). In mice, the sensory epithelium of the VNO is segregated into at least two structurally and functionally distinct layers: an apical layer that contains sensory neurons (VSNs) expressing the G protein G12i2 and members of the V1r family of vomeronasal receptors, and a basal layer that contains VSNs expressing G13i3 and members of the V2r receptor family (Buck 2000; Halpern and Martínez-Marcos 2003; Mombaerts 2004; Ryba and Tirindelli 1997). These distinct populations of VSNs have been hypothesized to detect and process different classes of chemical signals (Kimoto et al. 2005; Leinders-Zufall et al. 2000, 2004) and are likely to use different signal transduction mechanisms (Kelliler et al. 2006; Leyhold et al. 2002). This dichotomy is maintained in the accessory olfactory bulb (AOB) where apical VSNs project their axons to the rostral part of the AOB, whereas basal VSNs innervate the caudal AOB (for reviews see Buck 2000; Dulac and Torello 2003; Mombaerts 2004). Although it is generally difficult to predict with certainty which layer of the VNO a given VSN belongs to because of the diffuse boundary between the zones (Leinders-Zufall et al. 2004; Martini et al. 2001), cells are clearly identifiable in gene-targeted strains of mice in which individual VSNs express green fluorescent protein (GFP) under the control of a known V1r or V2r receptor.

Over the past decade substantial progress has been made toward understanding of the molecular logic and coding strategies in the mammalian vomeronasal system (Bozza et al. 2004; Del Punta et al. 2002a,b; Dulac and Torello 2003; Luo et al. 2003; Rodriguez et al. 1999). However, surprisingly little is known about the detailed biophysical properties of individual VSNs and how they transmit olfactory information to the AOB by spiking in response to sensory stimulation (Holy et al. 2000; Inamura et al. 1997, 2000; Leinders-Zufall et al. 2000, 2004). Earlier, dissociated mouse VSNs were used to investigate the major voltage-dependent conductances (Fieni et al. 2003; Liman and Corey 1996), and it was suggested that differences between voltage-dependent conductances in apical and basal VSNs may provide the initial basis for differences in excitability between the two types of VSNs in rodents. Recently, an acute mouse VNO slice preparation was used to measure and model the excitability of basal VSNs due to voltage-dependent Na+ and K+ currents (Shimazaki et al. 2006). Here, we sought to extend that work by characterizing the electrophysiological properties of both basal and apical mouse VSNs using transgenic mice in which VSNs express GFP under the control of either the V1rb2 or V2r1b receptor gene, and by focusing also on the voltage-gated Ca2+ (Cav) conductances that are important for spike generation and maintenance of persistent firing in VSNs. We show that coupling between the L-type Cav current and the BK large-conductance K+ conductance repolarizes spikes leading to prolonged firing in basal VSNs.

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and controls the persistent firing of the cells, suggesting a novel mechanism for regulating and encoding neuronal activity in the mammalian accessory olfactory system.

METHODS

Slice preparation

To clearly distinguish between VSNs of the apical or basal layer of the VNO epithelium, we used two mouse strains that harbor a targeted mutation in either the V1rb2 or the V2rb1 locus, resulting in cotranslation of tau-GFP along with V1rb2 or V2rb1 from a bicistronic message (Del Punta et al. 2002b; Rodriguez et al. 1999). Axons from these two distinct neuronal populations project to the anterior and posterior portions of the accessory olfactory bulb, respectively (Del Punta et al. 2002b; Rodriguez et al. 1999). All experiments were performed on 2- to 6-mo-old male or female mice. Original transgenic animals were generated in a mixed (129 × C57BL/6) background and then were backcrossed at least four times to the C57BL/6 background (Rodriguez et al. 1999). The procedures were carried out in accordance with protocols approved by the Universities of Maryland and Saarland. Acute coronal VNO tissue slices were prepared as described previously (Leinders-Zufall et al. 2000, 2004; Lucas et al. 2003). The mice were killed by inhalation of carbon dioxide, decapitated, and the entire VNO capsule was removed (Leinders-Zufall et al. 2000, 2004). The VNO was dissected in oxygenated, ice-cold modified artificial cerebrospinal fluid (ACSF, 95% O2-5% CO2) containing (in mM): 120 NaCl, 25 NaHCO3, 5 KCl, 5 BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 1 MgSO4, 1 CaCl2, 10 glucose (osmolarity adjusted to 300 mOsm, pH 7.3). The dissected VNO was embedded in 3% low-melting-temperature agarose (Sigma) and coronal slices (250 μm) were prepared using a Vibratome (Pelco101, model 1000). After sectioning, slices were transferred to a recording chamber and kept under continuous flow (1–2 mL/min) of oxygenated ACSF or remained on ice in oxygenated medium until needed (for ≤4 h). All experiments were performed at ambient temperature (20–22°C).

Electrophysiological recording

GFP-expressing (GFP+ ) VSNs were visualized in intact tissue slices with a ×40 water immersion objective lens (Olympus) using fluorescent illumination and a GFP filter set attached to the microscope (BX50WI, Olympus). These identified GFP+ neurons were inspected carefully to ensure that their dendrite and axon were not damaged during the slicing procedure. As a control, we also recorded from regular nonfluorescent (GFP−) neurons from the same V2r1b- or V1rb2-tau-GFP mice. Somatic recordings were made using patch pipettes pulled from a standard borosilicate glass (OD 1.5 mm; WPI, Sarasota, FL) on a vertical micropipette puller (PP-830, Narishige Instruments, Tokyo, Japan) followed by fire polishing. To assess the spontaneous firing properties of the VSNs under noninvasive conditions, we used extracellular loose-patch recording in some experiments. In this case, the pipette solution contained (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 10 HEPES (pH 7.4, 300 mOsm). The electrode solution used for recording of Cav currents contained (in mM): 10 Ca(OH)2, 110 NMDG, 140 methanesulfonic acid, 10 TEA-Cl, 10 HEPES (pH 7.4, 290 mOsm). In a subset of the experiments, extracellular Ca2+ was isotonically replaced with 20 mM Ba2+. The electrode solution used for recording of Cav currents contained (in mM): 140 Cs-methanesulfonate, 1 EGTA, 0.5 GTP Na-salt, 2 ATP-Mg salt, 10 HEPES (pH 7.1, 290 mOsm). Data were corrected off-line for a liquid junction potential of +4 mV for Nav and Kv currents and +11 mV for Cav currents.

Data analysis

Ionic currents were analyzed using PulseFit 8.54 (HEKA) and IGOR Pro 4.09 software. Voltage-activated currents were leak subtracted. Cells exhibiting leak currents >10 pA were not included in this analysis. Cell capacitance (Cm) was monitored using the automated function of the EPC-9 amplifier. A stable Cm-value over time was an important criterion for the quality of an experiment. Activation and inactivation curves of ionic currents were fit by the Boltzmann equation, giving values for the midpoint V1/2 (voltage at half-activation) and slope k where I(V) = I0[1 + exp [(V - V1/2)/k]]. These values are listed in Table 1 with the following parameters: slope k (slopeactiv or slopeinactiv) and V1/2 (V1/2activ or V1/2inactiv). Spike analysis was done off-line using IGOR Pro 4.09 software together with custom-written macros or the Neuromatic 1.86 software package (written by Jason Rothman, available at http://www.neuromatic.thinkrandom.com). Instantaneous spike frequency was measured as the reciprocal of the interspike intervals. Curves of the spike frequency as a function of injected depolarizing current were obtained by fitting the Michaelis–Menten equation to the data points, giving values for the maximal frequency Fmax and half-effective current I1/2, where F = (Fmax × f)/(I + I1/2). Statistical tests were performed using StatView 5.01 (SAS Institute, Cary, NC). The t-test (paired and unpaired) was used for measuring the significance of difference between two distributions. Multiple groups were compared using a one-way or two-way ANOVA. The Fisher’s least significant difference (LSD) test was used as a post hoc comparison of the ANOVA. If not otherwise stated results are presented as means ± SE.

Chemicals

All chemicals were purchased from Sigma unless otherwise stated. Drugs were prepared as stock solutions in DMSO or distilled water and diluted to the final concentration in ACSF for bath application. Peptide toxins were applied directly by the bath. Mibebradil and nimodipine were used to block T-type and L-type Cav currents, respectively. When used at micromolar concentrations, mibebradil preferentially inhibits T-type over other Cav channels (Martin et al. 2000; Randall and Tsien 1997). We applied this compound for a limited time only to avoid any side effects on other voltage-dependent channels. The peptide toxins α-conotoxin MVIIA and MVIC (both from Tocris, Ellisville, MO) were used to block N-type (Cav2.2) and N/P/Q-type (Cav2.1, 2.2) voltage-dependent Cav channels, respectively. Iberitoxin (Tocris) and charybdotoxin (Sigma) were used as specific blockers of large-conductance BK Ca2+ -activated K+ channels. Aminap and the synthetic nonpeptide inhibitor UCL1648 (both from Tocris) were used to assess the role of small-conductance SK Ca2+ -activated K+ channels. Final DMSO concentrations (≤0.1%, vol/vol) were tested in control solutions and had no effects.
**RESULTS**

**Patch-clamp recording from GFP-expressing VSNs in VNO tissue slices**

Individual, fluorescent VSNs were readily identifiable in the acute VNO slices prepared from the genetically altered mice (Fig. 1). With a combination of fluorescence or infrared differential interference contrast (IR-DIC) illumination (Fig. 1), patch-clamp recordings were obtained from such identified VSNs. The electrophysiological properties of V1rb2 (n = 53) and V2r1b (n = 66) neurons were then examined and compared with each other, as well as to some GFP− VSNs (n = 42). For these latter experiments, we chose VSNs that were located deep in the basal layer of the VNO sensory epithelium so that they would most likely represent basal VSNs that were located deep in the basal layer of the VNO lamina (Fig. 1). Passive membrane properties and spontaneous activity

The electrical properties of V1rb2 and V2r1b cells were examined by whole cell voltage-clamp or current-clamp recording. In some cases, we used extracellular loose-patch recording to measure spontaneous action potential firing rates under less invasive conditions. With KCl intracellular solution, both types of VSNs exhibited rather negative resting membrane potentials, averaging −74.3 ± 0.6 mV (n = 19) in V1rb2 and −75.3 ± 0.4 mV (n = 43) in V2r1b cells. GFP− cells had very similar values (−76.8 ± 0.9 mV; n = 17). Thus VSN resting potentials in the VNO slice, irrespective of whether GFP is overexpressed, are significantly more negative than those reported for freshly dissociated mouse VSNs (Fieni et al. 2003; Liman and Corey 1996). These values are also more negative than in a previous report that used acute VNO slices (Shimazaki et al. 2006).

We compared input resistance (Ri) and cell capacitance (Cm) among V1rb2 and V2r1b cells and found no significant differences between the two cell types (V1rb2: Ri = 1.9 ± 0.2 GΩ, n = 16; Cm = 6.8 ± 0.3 pF, n = 16; V2r1b: Ri = 1.8 ± 0.2 GΩ, n = 14; Cm = 6.4 ± 0.2 pF, n = 15). t-test: P = 0.40 for Cm and P = 0.59 for Ri. GFP−: Ri = 2.0 ± 0.1 GΩ, n = 10; Cm = 7.1 ± 0.5 pF, n = 18). These values are broadly consistent with previously reported results from mouse VSNs (Fieni et al. 2003; Liman and Corey 1996; Shimazaki et al. 2006). Together, these results indicate that VSNs expressing tau-GFP can be considered healthy by electrophysiological standards and should serve as an excellent model for the investigation of vomeronasal signaling mechanisms.

GFP− as well as GFP+ VSNs in the slice preparation were both spontaneously active at rest. This was observed by using either noninvasive, extracellular loose-patch recordings, or whole cell current-clamp recordings (at zero holding current). In all, 39 GFP+ cells were analyzed with respect to spontaneous firing. The vast majority of these cells (33/39, 84.6%) showed relatively low spontaneous firing frequencies (F0) averaging 0.29 ± 0.12 Hz in 12 V1rb2 cells and 0.26 ± 0.07 Hz in 21 V2r1b cells, respectively. However, a subset of these cells (6/39, 15.4%) encompassing both VSN types exhibited an enhanced rate of resting activity, averaging 3.0 ± 0.7 Hz. This phenotype was correlated with leak currents (>10 pA and elevated resting potentials. In 17 GFP− VSNs, we found an average resting activity of 0.35 ± 0.07 Hz. None of these cells exhibited an enhanced spontaneous firing rate. Therefore we limited our study to VSNs exhibiting low resting frequencies.

![Fig. 1. A and B: fluorescence images (pseudocolor) of acute, coronal vomeronasal organ (VNO) tissue slices (250 μm thick) showing the spatial location of V1rb2-tau-GFP (A) and V2r1b-tau-GFP vomeronasal sensory neurons (VSNs) (B) within the sensory epithelium. Each image was reconstructed by merging 6 individual optical sections obtained by confocal imaging. Each optical section had a thickness of about 5 μm. C–H: high-power micrographs of VNO slices showing individual V1rb2- (C–E) or V2r1b-expressing cells (F–H). Transmitted light images (C, F) and respective green fluorescent protein (GFP) fluorescence images (grayscale; D, G) were merged to show the position of the labeled cells (E, H). All VSNs analyzed in this study exhibited an intact cytoarchitecture including a thick dendrite projecting to the lumen (L) and ending in a knoblike swelling, and a thin axon projecting toward the basal lamina. Patch electrode, P.](http://jn.physiology.org/doi/fig/10.1152/jn.01492.2006)
FIG. 2. Comparison of voltage-gated inward and outward currents in V1rb2- and V2r1b-expressing VSNs. A: representative families of whole cell currents to a series of depolarizing voltage steps (as indicated in the figure) recorded from V1rb2- (top) and V2r1b cells (bottom), respectively. VSNs were exposed successively to extracellular bath solution (control), bath solution containing Cd2+ (100 μM), and bath solution containing Cd2+ (100 μM) and tetrodotoxin (TTX, 2 μM). Voltage-activated K+ channels were blocked by using a Cs+-based pipette solution. B: current density–voltage curves were constructed using peak amplitudes of the currents shown in A (with Cd2+). Plotted data are means ± SE. In this and all subsequent figures, the symbols (open and closed circles, closed triangles) will denote the 3 sampled populations of neurons. On average, peak current density was approximately 2-fold greater in V2r1b cells (closed circles; n = 7) vs. V1rb2 cells (open circles; n = 8). Data from GFP+ VSNs (closed triangles; n = 7) are shown for comparison. C: activation curves of the normalized voltage-gated Na+ (Nav) conductance derived from the data in B were similar in all 3 VSN populations. D: steady-state inactivation of the Nav currents was assessed by stepping membrane voltage to different prepulse voltages (from −120 to −20 mV) and measuring peak current amplitudes during a 10-ms step to −30 mV. Half-inactivation voltage of V1rb2 VSNs was shifted to more negative values than that of V2r1b and GFP+ VSNs. Data in both sets were fitted by the Boltzmann equation with parameters given in supplemental Table I. E: representative families of whole cell outward currents (in the presence of TTX (2 μM) and Cd2+ (100 μM)) to a series of depolarizing voltage steps (as indicated in the figure) recorded from V1rb2- (top) and V2r1b cells (bottom), respectively. K+-based pipette solution. F: current density–voltage plots obtained from families of voltage-gated K+ (Kv) currents as shown in E. G: activation curves of the normalized Kv conductance derived from the data in F were indistinguishable in all 3 neuronal populations.

Voltage-gated Na+ and K+ currents

V1rb2 and V2r1b cells both possessed large, rapidly activating and inactivating voltage-gated Na+ (Nav) inward currents (usually >1 nA) in response to a series of step depolarizations (Fig. 2A). These currents were recorded with Cs+-based intracellular solution to block outward K+ current. Because VSNs also showed robust voltage-gated Ca2+ (Cav) currents (see following text), we suspected that a portion of the inward current could be due to currents through Cav channels. Indeed, when we added Cd2+ (100 μM) to the bath solution, peak inward currents were reduced in both cell types, by as much as 4–16% (n = 14) (Fig. 2A). The remaining currents were fully abolished by application of tetrodotoxin (TTX, 2 μM), indicating that they were due to currents through Nav channels (Fig. 2A). It was previously reported that Nav currents in VSNs of the apical layer are larger than those of basal VSNs and that this phenotype can serve to distinguish between the two cell types (Fieni et al. 2003). By contrast, we found that V2r1b VSNs possessed much larger Nav currents than V1rb2 cells (Fig. 2A). This finding was further substantiated by analyzing current density (peak current normalized to cell capacitance) as a function of voltage in both cell types (Fig. 2B). On average, peak current density was approximately twofold greater in V2r1b (−274 ± 17 pA/pF, n = 7) than in V1rb2 cells (−126 ± 16 pA/pF, n = 9; t-test: P < 0.0001). We compared activation and inactivation parameters of Nav currents in both VSN types (Fig. 2, C and D). Whereas activation curves were nearly indistinguishable, there was a significant difference in half-inactivation voltage, with −65.7 ± 2.4 mV (n = 14) in V1rb2 versus −53.5 ± 0.8 mV (n = 15) in V2r1b cells (t-test: P < 0.0001). Detailed parameters obtained from Boltzmann fits of the activation and inactivation curves of Nav currents are listed in supplemental Table I.1

In the presence of TTX and Cd2+ and with intracellular KCl solution, V1rb2 and V2r1b cells both possessed prominent outwardly rectifying K+ currents in response to step depolarizations (Fig. 2E). These currents showed delayed rectifier gating properties typical of members of the Kv family of voltage-gated K+ channels. With Cd2+ in the bath, currents through Ca2+-activated K+ channels were absent, due to blockade of Ca2+ entry through Cav channels (see following text, Fig. 9). Kv current density in V2r1b cells was slightly larger than in V1rb2 cells (Fig. 2F). Mean current density (at 60 mV) was 176.6 ± 16.1 pA/pF (n = 7) in V2r1b versus 133.7 ± 9.9 pA/pF (n = 8) in V1rb2 cells (t-test: P < 0.05).

Repetitive firing in VSNs involves spike-frequency adaptation

A characteristic property of both freshly dissociated mouse VSNs (Liman and Corey 1996) and those maintained in VNO slices (Shimazaki et al. 2006) is that injection of only a few
picoamperes of inward current leads to repetitive action potential firing. Figure 3A shows that this specific feature was also observed in GFP VSNs. Under current clamp, both V1rb2 and V2rb1 cells generated repetitive action potentials in response to depolarizing 2-s current steps of 2 to 10 pA (Fig. 3A). Resting membrane potential refers to 0-pA current injection. A plot of mean instantaneous spike frequency as a function of injected current revealed that, in response to the same stimulus, V2rb1 cells generated considerably more spikes than V1rb2 cells, at least in the mid- to high range of the curves (Fig. 3B). These curves were well fitted by a Michaelis–Menten equation, showing saturation >20 pA with \( F_{\text{max}} = 11.2 \pm 0.5 \text{ Hz} \) (\( n = 6 \)) in V1rb2 and \( F_{\text{max}} = 19.1 \pm 1.6 \text{ Hz} \) (\( n = 5 \)) in V2rb1 cells. Larger Nav currents could lead to larger spikes in V2rb1 VSNs. Indeed, we found that V1rb2 cells generated much broader and smaller spikes than V2rb1. Spike duration (SpD) was measured as the width at 50% of the amplitude and spike amplitude (SpA) as the distance between the threshold and the peak (Fig. 3, C and D) with the following results: V1rb2: \( \text{SpD} = 15.8 \pm 0.7 \text{ ms}, \text{SpA} = 37.9 \pm 2.8 \text{ mV} \) (\( n = 53 \) spikes from 9 cells); V2rb1: \( \text{SpD} = 11.1 \pm 0.3 \text{ ms}, \text{SpA} = 64.8 \pm 1.2 \text{ mV} \) (\( n = 62 \) spikes from 10 cells). Injection of hyperpolarizing currents of \(-2 \) to \(-8 \text{ pA} \) revealed the presence of a hyperpolarization-activated “sag” in both cell types (Fig. 3A) which is typical of the activation of H-currents described in rat VSNs (Trotier et al. 1998). Both cell types produced rebound spikes during depolarization from hyperpolarizing current steps (Fig. 3A).

On the basis of anatomical and physiological evidence, chemical cues, once present in the VNO lumen, might be capable of stimulating VSNs for extended periods of time. For example, recordings from mitral cells in the accessory olfactory bulbs of behaving mice have revealed prolonged excitatory responses lasting for tens of seconds (Luo et al. 2003). These unusually long lasting responses could well be mediated by prolonged sensory input from VSNs. Therefore we investigated firing behavior of V1rb2 and V2rb1 cells in response to prolonged, depolarizing current steps (Fig. 3, E–G). Representative recordings to 10-s steps from both cell types are shown in Fig. 3E. These experiments revealed two unexpected results.

First, an important difference between the two cell types was that V1rb2 cells exhibited a tendency to oscillate and produce broad spikes with complex waveforms under these conditions,
whereas V2r1b cells were capable of maintaining persistent firing. Second, we observed profound spike-frequency adaptation in both cell types, in contrast to the general view that spike-frequency adaptation is absent in VSNs (Liman and Corey 1996). Because of the exceptional regularity of firing in V2r1b cells, we analyzed spike-frequency adaptation in these cells in more detail (Fig. 3F). Over the course of a 10-s current step, the frequency declined from 7.4 ± 0.8 to 3.1 ± 0.4 Hz (n = 6). Spike frequency–time plots were well fitted with a monoexponential function yielding an average decay time constant τ = 2.4 ± 0.3 s (n = 6). Profound spike-frequency adaptation occurred also in GFP+ VSNs (Fig. 3G). Here, with 60-s current steps, the frequency declined from 6.7 ± 0.4 to 1.3 ± 0.2 Hz (n = 5). With these even longer pulses, spike frequency–time plots were fitted best with two exponentials (τ1 = 2.36 ± 0.96 s; τ2 = 36.6 ± 14.9 s; n = 5) (Fig. 3G).

**Regenerative calcium spikes in VSNs**

Given the relatively long action potential durations observed in both types of VSNs and the appearance of oscillatory, complex spikes in at least the V1rb2 cells, we suspected that VSNs are capable of generating calcium spikes that might underlie some of these properties. This was tested by examining voltage responses to depolarizing current steps (10 s, 10 pA) in the presence of TTX (Fig. 4). Remarkably, application of 2 μM TTX, which is sufficient to eliminate all Nav currents in these cells (Fig. 2), did not completely disrupt spike generation. Instead, this treatment both reduced the firing frequency and dramatically broadened individual spikes compared with control recordings in both V1rb2 and V2r1b cells (Fig. 4, A–C). After TTX treatment, mean instantaneous spike frequency changed from 5.3 ± 0.3 to 3.2 ± 0.2 Hz in V1rb2 (n = 6) and from 6.5 ± 0.7 to 3.7 ± 0.4 Hz in V2r1b cells (n = 9). At the same time, spike duration (width at 50% of spike amplitude) increased from 23 ± 5 to 91 ± 15 ms in V1rb2 (n = 6) and from 12 ± 1 to 44 ± 8 ms in V2r1b cells (n = 9). In both cell types, TTX-resistant spikes showed profound spike-frequency adaptation (Fig. 4A).

Properties of these TTX-resistant regenerative spikes were somewhat reminiscent of low-threshold calcium spikes described in a variety of CNS neurons (Golding et al. 1999; Perez-Reyes 2003). When we applied Cd2+ (100 μM) to the bath solution, repetitive spiking was fully abolished, leaving only a single and strongly diminished initial spike followed by tonic membrane depolarization (Fig. 4A, TTX/Cd2+; n = 15). Subsequent experiments using inhibitors of Cav channels established that the TTX-resistant spikes indeed were caused by the activation of T- and L-type Cav channels (see Fig. 7) and thus represent regenerative calcium spikes.

**Presence of L-, T-, and N/P/Q-types of Cav channels**

Given the results of Figs. 3 and 4, we hypothesized that calcium spikes may underlie VSN action potential firing. To investigate this, it was necessary to first gain a detailed understanding of Cav conductances and their pharmacological properties in GFP+ and GFP− neurons. Mouse VSNs express at least two current components related to the activity of Cav channels: a low-voltage–activated T-type current and an L-type current (Fieni et al. 2003; Liman and Corey 1996). In accord with these studies, we consistently found a TTX-resistant inward current (2 μM TTX, Cs+–based intracellular solution) in GFP− VSNs that was activated by voltage steps from −100 to 0 mV and showed relatively slow inactivation kinetics typical of high-voltage-activated Cav currents (Fig. 5A, inset). Even under normal physiological conditions with 1 mM external Ca2+, the average amplitude of this current exceeded −50 pA. Intriguingly and in contrast to previous work using isolated VSNs (Fieni et al. 2003; Liman and Corey 1996), there was relatively little rundown of this current in the slice preparation over the course of 20 min (Fig. 5A), which enabled pharmacological dissection of its individual components.

Using the same voltage protocol but with 20 mM Ba2+ as the charge carrier, we found that this current was reduced to 71.8 ± 14.9% of control (n = 4) by application of mibebradil (Mib, 10 μM), which selectively blocked native T-type Cav current over the L-type current in rat retinal bipolar cells (Pan et al. 2001) (Fig. 5B). The remaining current was suppressed to 18.1 ± 2.4% of control (n = 6) by nimodipine (Nim, 5 μM), indicating that it was mostly due to L-type Cav channels (Helton et al. 2005) (Fig. 5B). Thus T- and L-type Cav channels appeared to be the main ion channels underlying this current. To confirm this, we applied a voltage protocol that favored selective activation of T-type currents (Fig. 5C). Indeed, under these conditions nimodipine had relatively little effect on Cav currents (89.4 ± 10.0% of control, n = 9), whereas the addition of mibebradil diminished the currents to 25.5 ± 2.0% of control (n = 5), leaving only a relatively small residual component. Thus although not specific in absolute terms, these two blockers nonetheless should be useful in dissecting the roles of T- and L-type channels in intact VSNs.
MVIIC (1 μM VSNs are likely to express members of Cav1, Cav2, and Cav3 channel families, with L- and T-type channels as the most prominent ones.

We next compared L- and T-type Cav currents in V1rb2 and V2rb1 cells (2 μM TTX, Cs⁺-based intracellular solution; Fig. 6). These experiments used 10 mM Ca²⁺ as the charge carrier. Figure 6A (control) depicts raw traces of families of currents in response to a series of step depolarizations from −120 mV to voltages between −80 and 40 mV in V1rb2 and V2rb1 cells, respectively. To isolate L-type currents, we applied nimodipine (Nim, 5 μM, Fig. 6A) and constructed current density–voltage plots of the nimodipine-sensitive component measured at the end of each voltage step (Fig. 6E). Unexpectedly, these currents activated at relatively negative potentials, between −60 and −50 mV, and reached a maximum between −10 and 0 mV (Fig. 6E). These properties suggest that both L- and T-type Cav channels could underlie low-threshold calcium spikes and contribute to action-potential–dependent Ca²⁺ entry in VSNs. The density of L-type currents was

![Image](https://via.placeholder.com/150)

FIG. 5. VSNs express multiple types of voltage-gated Ca²⁺ (Cav) currents. A: high-voltage–activated (HVA) Cav currents recorded in 5 GFP⁺ cells under normal ionic conditions (1 mM Ca²⁺ in the bath) showed little rundown over time; 200-ms-long voltage steps were applied every 20 s and mean current was measured at the end of each step. Peak amplitudes were normalized to control values obtained immediately after establishing the whole cell configuration at time 0. Inset: examples of original currents recorded at different time points (a, b, c). B: pharmacological dissection of individual Cav currents in GFP⁻ cells using 20 mM Ba²⁺ as charge carrier. Stepping the membrane voltage from conditioning −100 to 0 mV for 500 ms activated a slowly inactivating inward current (Ctrl, thick line); 10 μM mibefradil (Mib) slightly inhibited this current, whereas addition of nimodipine (5 μM, Mib/Nim) almost completely suppressed it. C: a low-voltage–activated (LVA) T-type Cav current was observed by stepping a conditioning 300-ms prepulse from −120 to −40 mV for 200 ms (Ctrl, thick line). Nimodipine had little effect on this current, whereas the addition of mibefradil significantly reduced it within 3 min, leaving only a small residual component. D: mibefradil/nimodipine-insensitive current (Mib/Nim, thick line) was blocked by ω-conotoxin MVIIIC (1 μM, CTx), indicating the presence of N/P/Q HVA Cav currents. Addition of Cd²⁺ completely suppressed any remaining current.

The nimodipine/mibefradil-insensitive current was further suppressed to 21.5 ± 6.1% of control (n = 5) by ω-conotoxin MVIIIC (1 μM, CTx), indicating that it was likely mediated by P/Q- and N-types of Cav channels (Catterall et al. 2005) (Fig. 5D). A second peptide toxin, ω-conotoxin MVIIIA, which specifically blocks N-type Cav channels, was less potent in suppressing this current (data not shown). Figure 5D shows that there was also a ω-conotoxin MVIIIC-insensitive but Cd²⁺-blockable, residual component that could be due in part to the activation of R-type Cav channels [but see Lipscombe et al. (2004) on the drug resistance of L-type Cav1.3]. Thus individual VSNs are likely to express members of Cav1, Cav2, and Cav3 channel families, with L- and T-type channels as the most prominent ones.
significantly larger in V2r1b (−15.2 ± 1.3 pA/pF, n = 7) than in V1rb2 cells (−8.9 ± 1.9 pA/pF, n = 7; t-test: P < 0.05).

T-type Cav currents were isolated by applying a mixture of nimodipine (5 μM) and ω-conotoxin MVIIC (Nim/CTx, 1 μM, Fig. 6A) and current density–voltage plots were constructed from peak values of the residual currents (Fig. 6B). These currents activated near −80 mV and reached a maximum between −40 and −20 mV. Current density was slightly larger in V2r1b (−6.9 ± 0.1 pA/pF, n = 7) than in V1rb2 cells (−5.5 ± 0.4 pA/pF, n = 7; t-test: P < 0.05). Notably both the T- and L-type Cav currents had no significant differences in the voltage dependence of current activation and inactivation between the two cell types (Fig. 6, C, D, F, and G; Table 1). Thus both cell types express L- and T-type Cav currents with very similar properties, but L-type currents are much more prominent in V2r1b cells than in V1rb2 cells.

**Calcium spikes depend on L- and T-type Cav channels in V1r and V2r cells**

Having provided evidence for L- and T-type Cav channel subtypes in V1rb2 and V2r1b cells, we next assessed the extent to which these channels were involved in the generation of calcium spikes in each cell type. A single action potential was evoked by a brief depolarizing current step (6 pA, 200 ms) (Fig. 7A). Addition of TTX (2 μM) revealed, in each cell type, the presence of TTX-insensitive spikes characterized by an increased delay and duration as well as a reduced amplitude compared with Na+-dependent action potentials (Fig. 7B, A and B). When we applied nimodipine (5 μM) these spikes were fully abolished in V1rb2 cells (n = 5). However, Ca2+ spikes in V2r1b cells were still present after this treatment with further increased duration (LSD: P < 0.01) and diminished spike amplitude (LSD: P < 0.025). These results provide evidence that, in at least the V2r1b cells, L-type Cav channels are involved in both determining the threshold of Ca2+ spikes as well as contributing to their repolarization phases. Subsequent application of mibefradil (10 μM) fully abolished calcium spikes in V2r1b cells (Fig. 7, A and B).

Finally, we assessed the role of Cav currents in controlling spike firing when Na+ channels are not blocked with TTX. Both nimodipine (5 μM) and mibefradil (10 μM) were applied in a mixture (Fig. 7C). Basal GFP− VSNs (n = 8) were depolarized with steps of current until a single spike was fired. The inhibitor mixture reduced spike amplitude (control: 65.1 ± 1.9 mV; inhibitors: 52.4 ± 2.7 mV), increased spike width (control: 8.2 ± 0.5 ms; inhibitors: 11.5 ± 1 ms), and also increased the delay of spike firing (control: 115.6 ± 7.4 ms; inhibitors: 164.5 ± 11.8 ms) (Fig. 7D), thus clearly indicating an essential role of the T-type and L-type Cav channels in action potential control.

**L-type Cav channels are essential for maintaining persistent spike firing**

We next asked whether Cav channels are involved in VSN action potential firing. Figure 8A shows a representative experiment (n = 4) in which persistent action potential firing was induced by current injection in a GFP− neuron. On the basis of the firing pattern, this cell is clearly identifiable as a basal VSN. We sequentially applied ω-conotoxin MVIIC (1 μM), mibebradil (10 μM), and nimodipine (5 μM) and monitored firing behavior under these conditions. Whereas ω-conotoxin MVIIC and mibefradil had no obvious effect on sustained action potential firing, nimodipine, strikingly, prevented maintained firing, leaving only a single action potential in response to the current step. These results suggest that maintained action potential firing was critically dependent on the activity of L-type Cav channels. This was further substantiated in experiments using V1rb2 (n = 4) and V2r1b cells (n = 5), in which application of nimodipine (5 μM) alone gave very similar results and effectively prevented maintained action potential firing in both cell types (Fig. 8B). The only significant difference with the experiment of Fig. 8A was that VSNs fired...
several action potentials before discharges finally stopped. Because T-type channels were already blocked in the experiment of Fig. 8A (bottom trace), this may indicate that T-type channels play a role in driving action potentials, especially at the beginning of the burst sequence, whereas the role of L-type channels becomes more pronounced after a few spikes.

Interplay between L-type Cav and BK channels underlies persistent action potential firing

Block of L-type Cav channels by nimodipine not only affected sustained action potential firing but also often caused action potential broadening and a loss of afterhyperpolarization, together with an increase in the interspike interval. This effect was particularly evident in V1rb2 cells, where the repolarization amplitude became progressively reduced within a few spikes (Fig. 8B, V1rb2), although a qualitatively similar effect was also observed in V2rb1 neurons (Fig. 8B, V2rb1). Recent evidence indicates that L-type Cav channels can form macromolecular complexes with large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK K\(_{Ca}\)) channels, producing Ca\(^{2+}\)/nanodomains through which Cav channels activate BK channels with submillisecond kinetics (Berkefeld et al. 2006). We hypothesized that, due to the effect of nimodipine, action-potential–induced Ca\(^{2+}\) entry through L-type Cav channels was significantly reduced or eliminated, potentially causing diminished activation of K\(_{Ca}\) channels and thus a loss of repolarization during repetitive action potential firing. If so, selective blockade of K\(_{Ca}\) channels should mimic the effect of nimodipine.

To test this, it was necessary to first gain insight into the properties of K\(_{Ca}\) currents in voltage-clamped V1rb2 and V2rb1 cells (Fig. 9, A and B). We focused on those K\(^+\) currents that depended on the activity of L-type Cav channels. Families of K\(^+\) currents were activated by voltage steps from −40 to 130 mV (Fig. 9A). In both cell types, this resulted in large outward currents that showed partial inactivation (Fig. 9A, control), in contrast to the outward currents that we observed in the presence of Cd\(^{2+}\) (compare Fig. 2E). We found that application of nimodipine (5 μM) caused a substantial reduction in the outward current amplitude (Fig. 9A, nimodipine), whereas application of either ω-conotoxin MVIIIC (1 μM) or mibebradil (10 μM) had very little or no effect (not shown). We plotted the nimodipine-sensitive component of the outward currents (Fig. 9A, subtraction) and constructed current density–voltage plots (Fig. 9B). These curves showed outward rectification and a characteristic N-shape, typical of K\(_{Ca}\) currents. Similar curves were obtained when we removed extracellular Ca\(^{2+}\) instead of adding nimodipine, confirming the dependence of these currents on Ca\(^{2+}\) entry (n = 3; Fig. 9B, control minus low Ca\(^{2+}\)). Thus it appears that activation of L-type Cav channels is selectively coupled to K\(_{Ca}\) currents in these cells. As with Nav and L-type Cav currents, the density of K\(_{Ca}\) currents (measured at 50 mV) was nearly twofold greater in V2rb1 (−109 ± 17 pA/pF; n = 11) than in V1rb2 cells (−47 ± 11 pA/pF; n = 5) (Fig. 9B).

![Figure 8](http://jn.physiology.org/)

**FIG. 8.** L-type Cav channels are necessary for the maintenance of persistent firing in the VSNs. A: effect of different Cav channel inhibitors were tested on the firing in basal GFP\(^+\) VSNs induced by a 10-s step of depolarizing current (10 pA, n = 4). ω-Conotoxin MVIIIC (CTx) and mibebradil (CTx/Mib) did not affect prolonged firing, whereas the addition of nimodipine (CTx/Mib/Nim) severely impaired persistent firing. B: representative recordings of the firing discharge induced by a 10-s step of depolarizing current (10 pA) in V1rb2 (n = 3) and V2rb1 (n = 4) VSNs before and after adding nimodipine to the bath. Nimodipine alone potently prevented persistent firing in both the VSN types, leaving only a few initial spikes at the beginning of the current injection.

![Figure 9](http://jn.physiology.org/)

**FIG. 9.** Specific coupling of large-conductance (BK) Ca\(^{2+}\)-activated K\(^+\) current to L-type Cav channels is critical for persistent firing in the VSNs. A: depolarizing 500-ms voltage steps from −40 to +130 mV were applied to V1rb2 and V2rb1 VSNs to measure net outward Kv currents. Recordings were obtained with KCl-based electrode solution without any additional inhibitors. Outward K\(^+\) currents in both types of VSNs were substantially reduced after treatment with nimodipine (5 μM) and subtraction yielded families of inactivating outward currents. B: in both V1rb2 and V2rb1 VSNs the current density–voltage curves derived from the peak amplitudes of the nimodipine-sensitive component had a characteristic N-shape (control minus Nimodipine).

In the experiment shown in the bottom graph (control minus low Ca\(^{2+}\)) we lowered the extracellular Ca\(^{2+}\) concentration to 1 μM instead of adding nimodipine. This revealed a component with the same basic properties (n = 3), demonstrating the Ca\(^{2+}\) dependence of the N-shaped conductance. C: similar N-shaped current density–voltage curves were also obtained after treatment with 2 specific inhibitors of BK channels, ibotenic acid (1 μM, n = 4, control minus Ibotoxin) and charybdotoxin (1 μM, n = 3, control minus Charybdotoxin). D: essential role of BK channels in persistent firing. Treatment with ibotenic acid (1 μM) disrupted persistent firing in VSNs in a manner very similar to the effect observed with nimodipine in Fig. 8 (n = 4). Inset: analysis of the width of the first spike in response to a 10-s current injection under control conditions (C, black bar) and after treatment with ibotenic acid (IBTs, gray bar). BK channel blocker caused significant spike broadening (t-test: **P < 0.01). No significant change occurred with respect to spike delay and amplitude (t-test: P > 0.2).
Are such $K_{Ca}$ currents caused by the activation of BK channels? This was tested in basal GFP+ cells by using subtype-specific blockers of $K_{Ca}$ channels (Fig. 9C). Using the same basic protocol as in Fig. 9A, families of outward $K^+$ currents were activated by voltage steps from $-40$ to $130$ mV. Instead of nimodipine, however, we now applied iberiotoxin ($1 \mu M$), a highly specific peptide inhibitor of BK$_{Ca}$ channels (Garcia et al. 2001; Wei et al. 2005). Remarkably, current density–voltage plots revealed that the iberiotoxin-sensitive component was nearly indistinguishable from the nimodipine-sensitive one ($n = 4$; Fig. 9C). A very similar effect occurred when we added charybdotoxin ($1 \mu M$, $n = 3$), another specific inhibitor of BK channels (Fig. 9C, control minus charybdotoxin). By contrast, application of highly selective inhibitors of small-conductance (SK) $K_{Ca}$ channels, UCL1648 ($1 \mu M$) or apamin ($250$ nM), failed to promote this effect (data not shown). Thus we conclude that activation of L-type Cav channels is specifically coupled to BK channels in these neurons.

These findings enabled us to test directly whether maintained action potential firing in VSNs depends on BK channel activation. To do this, we investigated the effect of iberiotoxin ($1 \mu M$) on spike discharges in current-clamped basal GFP+ VSNs (Fig. 9D). Strikingly, iberiotoxin prevented maintained spiking in a manner that was nearly indistinguishable from the nimodipine-induced effect ($n = 4$). For instance, there was a progressive reduction of the afterhyperpolarization, combined with considerable spike broadening during the first few action potentials and increased interspike interval before firing finally stopped (Fig. 9D). In these experiments, the average spike width increased from $7.8 \pm 0.9$ ms (control, C) to $10.2 \pm 1.1$ ms in the presence of iberiotoxin ($n = 4$; paired $t$-test, $P < 0.01$) (Fig. 9D, inset). No such effect could be induced by the SK channel inhibitors apamin ($250$ nM) or UCL1648 ($1 \mu M$) (not shown). Thus we conclude that action-potential–dependent $Ca^{2+}$ entry mediated by L-type Cav channels that couple specifically to BK channels is essential for maintaining action potential firing in VSNs.

**Discussion**

A primary goal of this study was to explore whether gene-targeted, identifiable VSNs can serve as a model for investigating signaling mechanisms in the mouse VNO. By selecting V1rb2-tau-GFP and V2r1b-tau-GFP VSNs as prototypical apical or basal VSNs, respectively, we have begun to systematically define the functional properties of sensory neurons located in each of the two primary expression zones of the VNO. Collectively, our results suggest a novel mechanism for regulating and encoding neuronal activity in the mammalian accessory olfactory system that depends specifically on the interplay between L-type Cav channels and BK channels. Potentially, these findings could be important for gaining a deeper understanding of the mechanisms that underlie long-term potentiation and pheromonal learning during social recognition in the accessory olfactory bulb (see following text and Kaba and Huang 2005).

**Nav current as a fingerprint of the VSN type**

We found that Nav currents recorded in apical V1rb2 VSNs were consistently smaller than those in the basal GFP+ and V2r1b cells. Nav currents were previously shown to differ in apical versus basal VSNs using a dissociated cell preparation. However, in that case the Nav currents were found to be smaller in the basal VSNs (Fieni et al. 2003). One reason for this discrepancy could be the truncation of axons during the dissociation procedure, leading to the loss of Nav channels. To avoid this problem, we visually inspected each GFP+ VSN before experimentation to ensure the integrity of the cytoarchitecture. Fieni et al. (2003) correlated dendritic length of VSNs with cell-type–specific immunolabeling and found that, on average, apical VSNs have significantly shorter dendrites than basal VSNs. Thus it has been assumed that apical VSNs express fewer Nav channels than basal VSNs. By contrast, Dean et al. (2004) found no correlation between the size of both the Nav and the Kv currents and VSN dendritic length. Both groups used mice of different strains: CD1 (Fieni et al. 2003) versus BALB/c and CBA mice (Dean et al. 2004). It is unclear whether these differences could be accounted for solely by strain differences. In fact, we recorded from several basal VSNs in slices obtained from CD1 mice and found Nav and Kv current densities similar to those reported earlier in V2r1b cells (unpublished data). In the intact VNO slice, both apical V1rb2 and basal V2r1b VSNs are located in partially overlapping regions of the VNO neuroepithelium. Therefore VSNs of both neuronal types exhibit dendrites of variable length. Recording from genetically labeled VSNs that are maintained in intact epithelial slices provides a means to ensure the morphological integrity of the cells and to unequivocally identify each VSN before physiological analysis.

**Basal V2r1b neurons are optimized for maintained action potential firing**

We asked whether larger Nav currents in V2r1b VSNs correlate with more efficient action potential generation. Both the V1rb2 and V2r1b VSNs showed input resistances in the range of several gigaOhms, similar to reported values in VSNs of different species (frog: Gjerstad et al. 2003; rat: Inamura et al. 1997; garter snake: Taniguchi et al. 2000; mouse: Liman and Corey 1996; Shimazaki et al. 2006). In both neuronal types, injection of only a few picoamperes of inward current was sufficient to elicit maintained spiking (Fig. 3). However, V2r1b VSNs were capable of firing at higher frequencies than were V1rb2 VSNs. Analysis of action potential waveforms showed that V2r1b cells produce faster and larger spikes than those of V1rb2 VSNs, in accordance with the presence of larger Nav and Cav currents in the V2r1b cells. A finding of importance was that V1rb2 and V2r1b cells differ also in their ability to fire action potentials in response to prolonged, depolarizing current steps. Whereas V1rb2 cells exhibited a tendency to begin to oscillate after a few seconds and produce broad spikes with complex waveforms under these conditions, V2r1b cells were exquisitely capable of maintaining persistent firing for tens of seconds. This remarkable regularity is likely to underlie the prolonged excitatory responses that have been recorded from some mitral cells in the accessory olfactory bulb of behaving mice (Luo et al. 2003). In this respect, it is interesting to note that long-term potentiation at the mitral-to-granule cell synapse in the accessory olfactory bulb, which might underlie pheromonal learning in the context of selective pregnancy termination (the Bruce effect), is effectively trig-
gered by low-frequency, 10-Hz pulses applied for extended periods of time (theta frequency stimulation; Kaba and Huang 2005). Thus mouse VSNs, especially those in the basal zone, are optimized to produce exactly the kind of firing properties that this form of cellular plasticity requires.

Cav currents underlie oscillatory calcium spikes and VSN action potential generation

Sustained Cav currents were a prominent component of the VSN whole cell current in the slice preparation. Surprisingly, these currents did not show much rundown over time, in contrast to Cav currents in dissociated VSNs (Fieni et al. 2003; Liman and Corey 1996). These remarkably stable Cav currents thus allowed us to assess the role of Cav channel subtypes in VSN action potential firing.

A previously unknown feature of mouse VSNs is their ability to generate slow, regenerative calcium spikes in response to membrane depolarization (Fig. 4). Low-threshold Cav conductances have been described to drive action potentials in CA1 pyramidal neurons (Golding et al. 1999) and to maintain dendritic calcium spike propagation in Purkinje neurons (Cavelier et al. 2002). We thus expected low-threshold T-type Cav currents to mediate calcium spikes in mouse VSNs as well. However, in at least the V1rb2 cells, calcium spikes were fully abolished after inhibition of L-type Cav channels with nimodipine. In the V2rb1 cells, block of L-type Cav current did not fully abolish the calcium spikes but further increased their duration and diminished their amplitude (Fig. 7). Here, inhibition of both the T-type and L-type Cav currents was required for complete suppression of calcium spikes. This differential inhibition may be well accounted for by differences in the size of Cav currents in both cell types (Fig. 6). Thus both types of Cav channels are required for shaping action potential properties in mouse VSNs. This notion was further strengthened by the fact that under normal physiological conditions (i.e., with Na+ channels not inhibited by TTX), a combination of mibefradil and nimodipine was required to affect action potential kinetics (Fig. 7).

T- and L-type currents contributed most to the net Cav conductance in mouse VSNs, with a relatively minor contribution from N/P/Q-type and possibly R-type Cav currents. The pharmacological profile of these Cav currents does not allow us to identify any particular Cav channel isoform with certainty (Catterall et al. 2005). However, a recent study using immunohistochemical and RT-PCR approaches revealed the presence of multiple Cav channel subunits including the L-type Cav1.2 (alpha1C) channel in mouse VNO (Murakami et al. 2006). A growing body of evidence indicates the presence of multiple isoforms of L- and T-type Cav channels in olfactory sensory neurons as well (Gautam et al. 2007). Both T-type and L-type Cav currents are known to activate fast enough to contribute to the spike firing in a variety of neuronal types (Helton et al. 2005; McCobb and Beam 1991). The role of T-type Cav channels is well established in setting the low-voltage spike threshold in mammalian neurons (Perez-Reyes 2003). Some neuronal L-type Cav channels, especially the Cav1.3 isoform, also activate at relatively low voltages sufficient to drive action potential firing and being involved in action-potential–dependent Ca2+ entry (Lipscombe et al. 2004). In olfactory sensory neurons, however, it is especially the T-type Cav current that sets the threshold for action potential firing (Kawai et al. 1996). Furthermore, T-type Cav channels are critical for propagation of a Ca2+ wave from the distal dendrite to the cell soma (Gautam et al. 2007).

In mouse VSNs, the T-type Cav current activates at around −80 mV (Fig. 6), close to the resting membrane potential of −75 mV, which is slightly more negative than the activation voltage for Nav currents (about −70 mV, Fig. 2). Interestingly, we also found relatively negative activation thresholds for L-type Cav currents (about −60 mV, Fig. 6). Therefore it appears that both T- and L-type Cav channels are essential for maximal excitability in mouse VSNs. The exact contribution of T- and L-type Cav channel subtypes in VSN spike generation and maintained rhythmic firing should be dissected in future experiments by using genetic models (e.g., see Chan et al. 2007), together with more elaborate spike waveform voltage command protocols (e.g., see Bean 2007).

Interplay between L-type Cav and BK channels is crucial for persistent VSN firing

A key finding of this communication, specifically in the basal VSNs, is a novel mechanism for regulating persistent neuronal activity in the accessory olfactory system, which depends on specific coupling of L-type Cav channels and BK channels. BK channels were previously shown in mouse brain to colocalize with both Cav1.2 and Cav1.3 L-type channels (Grunnet and Kaufmann 2004) and, more recently, Berkelfeld et al. (2006) reported clear evidence that Cav1.2 channels are capable of forming functional macromolecular complexes with BK channels in rat brain. We used established pharmacological tools to specifically assess the role of those Cav channels that might control persistent action potential firing in VSNs. Treatment with mibefradil and ω-conotoxin MVIIC demonstrated that T-type and N/P/Q-type Cav channels are not critically involved in this regulation. By contrast, application of nimodipine effectively disrupted persistent firing induced by membrane depolarization (Fig. 8). Thus an L-type Cav channel, most likely involving Cav1.3, is essential for maintained firing in these VSNs. This effect was reminiscent of the contribution of L-type Cav channels to maintained rhythmic firing in some invertebrate respiratory neurons (Spafford et al. 2006).

Nimodipine—but not mibefradil or ω-conotoxin MVIIC— inhibited outward K+ currents to the same extent as treatment with Cd2+ or removal of extracellular Ca2+ ions. These nimodipine-sensitive K+ currents were caused by the activation of BK channels because they were selectively inhibited by iberiotoxin, a specific peptide blocker binding to the pore-forming alpha subunit (Slo) of the BK channels (Garcia et al. 2001; Giangiacomo et al. 1992; Wei et al. 2005), but not by specific inhibitors of SK Ca2+-activated K+ channels such as UCL1648 and apamin. BK channels are thought to control spike repolarization, firing frequency, and spike-frequency adaptation (SFA) in many neurons by giving rise to the fast afterhyperpolarization (AHP) following a spike (Sah and Faber 2002). Similar mechanisms may also exist in mouse VSNs because iberiotoxin effectively disrupted persistent firing and increased the width of the initial spike. However, we also observed another component of the AHP with a substantially slower time course that was always present after termination of depolarizing current injections (see Figs. 3 and 8). This AHP...
was usually not affected by Cav channel inhibitors such as nimodipine or Cd\(^{2+}\). As discussed by Sah and Faber (2002) there might exist an as yet unidentified ion channel underlying K\(_{Ca}\)-independent AHPs.

**Spike-frequency adaptation: possible link to sensory adaptation**

Spike-frequency adaptation (SFA) is a fundamental feature of neural dynamics. In the VNO, it was generally thought that SFA is not present in VSNs. However, previous studies had injected current steps for only about 2–3 s (Liman and Corey 1996). By using prolonged current steps (10 or 60 s, respectively), we found that all three sampled VSN populations exhibit profound SFA. Various ionic currents modulating spike generation have been implicated to cause this type of neural adaptation including Kv currents (M-type currents), the cross talk of Ca\(^{2+}\) currents and intracellular Ca\(^{2+}\) dynamics with K\(_{Ca}\) channels (APH-type currents), persistent Nav currents, and slow recovery from inactivation of the fast Nav current (Benda et al. 2003; Miles et al. 2005; Zeng et al. 2005). In our case, it is unlikely that inactivation of the fast TTX-sensitive Nav channels solely mediates SFA because TTX-treated VSNs that generate pure Ca\(^{2+}\) spikes still exhibited this feature (Fig. 4). Treatment with inhibitors of SK K\(_{Ca}\) channels also did not affect SFA. Furthermore, we can exclude a role of T-type and N/P/Q-type Cav currents because their block had essentially no effect on SFA (Fig. 8A). However, a role of L-type Cav and BK channels could not be tested properly because blockade of both conductances suppresses repetitive firing. In olfactory sensory neurons, SFA is also present and has been proposed to be involved in sensory adaptation (Delgado et al. 2003; Kawai 2002; Narusuye et al. 2003). VSNs have been thought to lack any form of sensory adaptation (Holy et al. 2000). It remains to be seen how SFA affects the encoding of different classes of chemical cues in the VNO.

In summary, our study provides several novel ionic mechanisms for regulating neural activity in the accessory olfactory system that may have been overlooked previously due to potential rundown of Cav conductances in preparations of acutely isolated neurons. We expect that these findings are of direct relevance to future studies aimed at understanding how social cues are encoded in the mammalian vomeronasal system and how social memories are formed.

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