Heterogeneity of Voltage- and Chemosignal-Activated Response Profiles in Vomeronasal Sensory Neurons

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INTRODUCTION

Pheromones are a vehicle for chemical communication in vertebrates and serve to relay important information for mate selection, individual and gender identification, and recognition of reproductive and social status (Brennan and Keverne 2004; Dulac and Torello 2003; Gosling and Roberts 2001; Halpern and Martínez-Marcos 2003; Luo and Katz 2004; Wyatt 2003). Activation of cellular signaling cascades in the vomeronasal organ (VNO), combined with hormonal changes mediating reproduction, are driven by chemosignals that must be transduced from an external chemical message into an electrical signal read by the CNS (Dulac 2000; Zufall et al. 2002). Therefore the translation of information provided by the cellular transduction machinery of the VNO ultimately mediates the final change in behavior of the animal. We explored a novel vertebrate model (lizard; Liolaemus species) that shows quantifiable behavioral displays in response to pheromones (Labra and Niemeyer 2004; Labra et al. 2001, 2003) to ascertain whether electrophysiological properties in the vomeronasal sensory neurons (VSNs) could be measured in response to natural secretions used for such forms of chemical communication.

Recent advances in the molecular identities of VNO transduction proteins (Berghard et al. 1996; Dulac and Axel 1995; Liman et al. 1999; Rodriguez et al. 2002; Ryba and Tirindelli 1997), the application of neuronal imaging techniques to pan for pheromone-excitable cells (Boschat et al. 2002; Leinders-Zufall et al. 2000, 2004), the development of multielectrode recording arrays to screen VSN populations (Holy et al. 2000), the ability to make in situ recordings from the accessory olfactory bulb in the behaving, mating animal (Luo et al. 2003), and the behavioral phenotyping of transgenic mice deficient in VNO transduction channels and receptors (Del Punta et al. 2002; Leybold et al. 2002; Stowers et al. 2002), has deepened our understanding of the transduction events operational in the rodent VNO. Two families of G-protein-coupled receptors, V1R and V2R, are thought to bind chemosignals to activate two different respective G proteins, G\textsubscript{i2} and G\textsubscript{o2}, respectively (Berghard and Buck 1996; Berghard et al. 1996; Dulac and Axel 1995; Jia and Halpern 1996; Luo et al. 1994; Shinohara et al. 1992). The final event is mediated by the activation of a member of the transient receptor potential family (TRPC2) to complete the transduction into an electrical signal (Liman et al. 1999; Lucas et al. 2003).

Although a large proportion of the VNO transduction cascade has been discovered in rodents, there are some interesting physiological differences and technical advantages for using reptiles. First, in Sternotherus odoratus (stinkpot, musk turtle), we found that sexual dimorphism existed in the composition of the voltage-activated conductances, the size of the VN neurons, and in the GTP-binding distribution along the microvillar surface of the VN epithelium (Fadool et al. 2001; Murphy et al. 2001). The dimorphism in cellular transduction machinery may be a reflection of the dimorphism at the organism level; female S. odoratus are much larger in body size than males. Second, the anatomical and functional segregation of the two major VNO signal transduction pathways does not exist in other higher vertebrates and mammalian models; it is selectively operational in the order Rodentia. Goats, dogs, horses, snakes,
and turtles use only a single G-protein-coupled signal transduction pathway rather than an apical and basal distribution of the V1R and V2R family of receptors and related signaling molecules, respectively (Murphy et al. 2001; Takigami et al. 2000). In addition, it has been demonstrated that the reptilian VNO is not an organ used solely for pheromone transduction, but other important chemosignals, such as those produced by prey items, are encoded by the VNO (see review, Halpern and Martinez-Marcos 2003). Last, we found that the stimulus response rate for a reptilian VNO neuron to respond to at least one of five presented pheromone extracts was high (34–59%) (Fadool et al. 2001; this study) in comparison to that reported in rodents [1–3 (isolated pheromones) to 38% (dilute urine)] (Holy et al. 2000; Leinders-Zufall 2000).

The highly favorable pheromone response rate makes single-cell electrophysiology practical in the reptilian VNO. The Liolaemus lizard, in addition, possesses an array of quantifiable behavioral displays, such as scent marking, cloacal rubbing, forearm waving, tail shaking, and head bobbing, all of which it will undergo within the confines of a terrarium (Labra and Niemeyer 2004). Despite the wealth of ecological studies of chemical communication in this genus, amazingly little is known about the anatomy or physiology of the VNO in these organisms. To bridge the gap between reproductive-related behaviors and single-cell electrophysiology, we sought an initial characterization of the VNO of the Liolaemus lizard. It is one of our goals to utilize the discovered electrophysiological properties of this reptilian model as a future foundation on which to explore an array of social behaviors that may be seasonally or developmentally modified for an animal that lives in a chemically complex environment.

METHODS

Solutions and antibodies

The intracellular pipette solution contained (in mM) 100 KCl, 10 HEPES, 10 EGTA, 2 MgCl₂, 0.8 CaCl₂, 2.5 NaATP, 0.5 NaGTP, and 20 NaCl (pH 7.4; 233 mOsm). The nucleotides were prepared daily and added to the pipette solution just prior to recording. Recording bath solution (reptile Ringer solution) contained (in mM) 116 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 15 glucose, and 5 NaPyruvate (pH 7.4). Phosphate-buffered saline (PBS) contained (in mM): 10 Tris, 10 HCl, 10 glucose, and 5 NaPyruvate (pH 7.4). Homogenization buffer (HB) contained (in mM) 316.9 NaCl, 2.7 KCl, 10.2 Na₂HPO₄, and 1.8 KH₂PO₄ (pH 7.4). Homogenization buffer (HB) contained (in mM) 320 sucrose, 10 Tris base, 50 KCl, and 1 EDTA (pH 7.8). Protease inhibitor (PI) solution was added to HB just prior to use for a final concentration as follows: 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, and 10 mM Na₂VO₃. Tris stripping buffer (TSB) contained (in mM): 10 Tris, 10 β-mercaptoethanol, with 1% SDS (pH 8.8). Sodium citrate stripping buffer (SCSB) contained (in mM): 100 Na₂C₃H₅O₇, 2H₂O, 10 β-mercaptoethanol, with 1% SDS; pH 3.0.

The rabbit polyclonal antiserum anti-G₁α (1:950) and anti-G₁α_i-3 (1:500) were purchased from Santa Cruz (Santa Cruz, CA). Anti-G₁β was raised in rabbits immunized with a synthetic decapetide MSELDALRQE (amino acids 1–10 of bovine transducin β subunit). Anti-G₁α_i-3 was raised against a peptide corresponding to an amino acid sequence within a highly divergent domain of G₁α_i so that it would react with G₁α_i-3. Mouse monoclonal anti-G₁α_i (1:10,000) was purchased from BD Biosciences (San Jose, CA) and was generated against amino acids 161–171 of the human form of G₁α_i. Anti-G₁α_i-1 (1:1,000) was a generous gift from Dr. Albert Farbman (Northwestern University, Evanston, IL), who generated the antiserum equivalent to Reed’s antiserum (CY coupled to KTAEDQGVDEKEREA, near the amino terminus of rat G₁α_i (Jones and Reed 1989). Anti-G₁α_y (E973), made to amino acids 115–133 of the G₁α_y peptide sequence and anti-G₁α_i-1 (E976), made to amino acids 160–172 of the G₁α_i-1 sequence (Strathmann and Simon 1990), were gifts from Dr. John Exton (Vanderbilt University, Nashville, TN). Affinity-purified anti-G₁α_i (E973), a sequence conserved across species from human to snail (an amino terminal cysteine coupled to the last 12 amino acids: CKLQNLKKEYNLV) (Gutowski et al. 1991) was a gift from Dr. Paul Sternweiss (UT Southwestern, Dallas, TX). Horseradish-peroxidase conjugated donkey anti-rabbit (Amersham-Pharmacia, Arlington Heights, IL) and goat anti-mouse (Sigma-Aldrich, St. Louis, MO) were used as secondary antisera at 1:5,000—6,000.

Animal collection and maintenance

Two Liolaemus species (L. bellii and L. nigroviridis) were collected in Central Chile in the Andes Mountains (Farellones: 33°20’S; 70°19’W; 2,300 m), east of Santiago. Forty-two animals were collected during January 2003, representing the summer activity period of these species (Donoso-Barros 1966). Only adult animals were collected of known body size that fell within that reported for reproductive maturity, namely for L. bellii, the mean snout–vent length (svl) was 69.5 ± 1.5 (SE mm) (n = 18) for males and 64.0 ± 1.6 (n = 11) for females and for L. nigroviridis, the mean svl was 66.7 ± 2.1 (n = 8) for males and 54.7 ± 2.1 mm (n = 5) for females.

Lizards were transported to Florida State University (FSU), Tallahassee, FL, under approval of the U.S. and Chilean government regulations for animal importation and following inspection at the United States Agriculture, Fish, and Wildlife Division. Lizards were maintained in an indoor vivarium at the Biological Research Facility at FSU. Lizards were housed in glass terraria that were equipped with special lighting (Neodymium 150-W daylight Lamp; Exoterra No. PT-2114) to provide heat, periodicity of the normal light spectrum (12L:12D), and UVA. The mean temperature inside the terrarium was established at 33°C during the light period (L) and 23°C during the dark period (D). The terraria were equipped with 3-cm-deep walnut chips (ESU Reptile Desert Blend, Petsmart, Tallahassee FL), a bowl for water, and rocks/plastic boxes for basking and shelter, respectively. Water was supplied ad libitum, but food (mealworms or crickets) was provided every other day and dusted with vitamins (T-Rex Bio-Vite Plus, Ocean Nutrition, San Diego, CA) once per week.

Collection of natural body secretions

Collections of natural body secretions were taken from L. bellii. Precloacal plugs were harvested from the male lizards by gently squeezing the base of the tail near the ventral cloacal opening or slit (Escobar et al. 2001) (Fig. 7A). Because the energetic demands to synthesize this pheromone-containing plug requires ~2–3 mo duration, only one sample was taken per animal after its death for electrophysiology experiments (see following text). A ~2-cm² patch of dorsal and ventral skin was also harvested at the time of sacrifice from both sexes. Feces were collected from both sexes by isolating individuals in terraria to positively identify the deposition. Precloacal secretion, skin, and feces samples were homogenized in a Kontes tissue grinder (size 20) for 50 strokes on ice in 400 μl reptile Ringer. Samples were clarified by centrifugation (Eppendorf 5415, setting 10), and the supernatant was collected and stored until use at ~20°C. The body extracts used in this study were collected over the 2003 South American months (Jan–April) found to be the peak reproductive period of these species (Labra et al. 2003).
Vital dye labeling and cryosectioning

Procedures for animal care, handling, and killing were done in accordance with the National Institutes of Health and the American Veterinary Association (AVMA) as reviewed by the Animal Care and Use Committee (ACUC) of FSU. Lizards were immobilized and anesthetized using 4°C hypothermia for 20 min. Tetramethylrhodamine-conjugated dextran (10,000 MW neutral, Molecular Probes, Eugene, OR) was introduced into the VNO orifice using a handmade syringe pump that delivered a volume of 7 μl to the cavity (Fadool et al. 2001; Friedrich and Korshing 1997; Wachowiak and Cohen 1999). The dextran dye was diluted in PBS plus 0.5% Triton-X 100 to a final concentration ranging between 2 and 4% and was applied for a period of 20 min. The animal then recovered at room temperature for 30 min prior to reinsertion into the terrarium. After migration of the dye over a two week period, lizards were re-anesthetized and then were given a lethal injection of sodium pentobarbital (Butler, Columbus, OH) followed by decapitation. The lizard head was fixed with 4% formaldehyde (Mr 49,000–53,000; Sigma) and 5 μg/cm² laminin-coated (laminin-like engineered protein polymer, BD Biosciences, San Jose, CA) Corning dishes (Fisher No. 2500) (Leinders-Zufall et al. 1997). Isolated vomeronasal neurons were viewed at ×40 magnification (Axiovert 135, Carl Zeiss, Thornwood, NY) with Hoffman modulation contrast optics for patch-clamp recording (Hamill et al. 1981). Patch pipettes were fabricated from Jencons borosilicate glass (Catalog No. M15/10, Jencons, Bedfordshire, UK), fire-polished to ~1 μm (bubble No. 5.0) (Mittman et al. 1987), and coated near the tip with beeswax to reduce the pipette capacitance. Pipette resistances were between 7 and 10 MΩ; this produced high-resistance seals (between 8 and 14 GΩ) by applying gentle suction to the lumen of the pipette on contact with the cell. The access resistance was continuously monitored throughout an experiment and ranged from 1.8 to 14 MΩ. In all experiments, cells were voltage-clamped at a holding potential (Vh) of −60 mV unless specified otherwise.

Voltage- and chemical-activated currents were recorded in the whole cell configuration using an integrating patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA). The analog output was filtered at 5 KHz and digitally sampled every 100 μs for the acquisition of both voltage- and chemical-activated currents. Data acquisition and subsequent storage and analysis of the digitized records were carried out using pClamp8.09α software (Axon Instruments) in combination with the analysis packages Origin (MicroCal Software, Northampton, MA) and Quattro Pro (Borland International, Jericho, NJ). Data traces were subtracted linearly for leakage conductance. Sixty to 70% of the series resistance could be compensated using the Axopatch 200B. Capacitance was also compensated using the amplifier; any remaining transients were nulled, postrecording, using Origin. The inactivation of the macroscopic current, during a 400-ms voltage step from −90 to +40 mV, was fit to the sum of two exponentials by minimizing the sums of squares using a bi-exponential function (y = y0 + Ae−(x−x0)/τ1 + Ae−(x−x0)/τ2). The two inactivation time constants (τ1 and τ2) were combined by multiplying each by its weight (λ) and summing. The deactivation of the macroscopic current was fit similarly but to a single exponential (y = y0 + Ae−(x−x0)/τ). Differences between sexes in a particular biophysical property were analyzed by tmax test and then by Student’s t-test. No tests with unequal sample sizes violated homogeneity of variance (i.e., none failed the tmax test) and statistical significance in all tests was defined at the 95% confidence interval (Steel and Torrie 1980).

Electrophysiology

L. oliaimensis lizards were anesthetized and killed as in the preceding text. Neurons from the VNO were isolated by incubation in Ca²⁺-free, cysteine-activated papain (25 units; Worthington Biochemicals, Lakewood, CA) in reptile Ringer solution as previously described (Fadool et al. 2001). The resulting single vomeronasal cells were plated onto 0.01% poly-d-lysine-hydrobromide-coated (Mr 49,000–53,000; Sigma) and 5 μg/cm² laminin-coated (laminin-like engineered protein polymer, BD Biosciences, San Jose, CA) Corning dishes (Fisher No. 2500) (Leinders-Zufall et al. 1997). Isolated vomeronasal neurons were viewed at ×40 magnification (Axiovert 135, Carl Zeiss, Thornwood, NY) with Hoffman modulation contrast optics for patch-clamp recording (Hamill et al. 1981). Patch pipettes were fabricated from Jencons borosilicate glass (Catalog No. M15/10, Jencons, Bedfordshire, UK), fire-polished to ~1 μm (bubble No. 5.0) (Mittman et al. 1987), and coated near the tip with beeswax to reduce the pipette capacitance. Pipette resistances were between 7 and 10 MΩ; this produced high-resistance seals (between 8 and 14 GΩ) by applying gentle suction to the lumen of the pipette on contact with the cell. The access resistance was continuously monitored throughout an experiment and ranged from 1.8 to 14 MΩ. In all experiments, cells were voltage-clamped at a holding potential (Vh) of −60 mV unless specified otherwise.

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Chemosensory stimulation

Body secretions (chemicals) were puffer applied on the vomeronasal neurons for 700 ms from a seven-barrel glass micropipette (1.2 mm OD, No.17-12-M, Frederick Haer, Bowdoinham, ME) coupled to a pressurized valve system (Picospritzer, General Valve, Fairfield, NJ) (see Fadool et al. 1991). Presentation of secretions was applied in random sequence. In most trials, fluorescein was used as an indicator
in one barrel of the pipette, which was varied from one experiment to another, to position the tip of the pipette relative to the cell and to ensure that the delivered compound completely surrounded the cell and its associated processes. The magnitude of the response to 0.5 M KCl was found to be independent of which of the six barrels contained the depolarizing solution. Dilution of the chemical between the pipette and the cell surface, an average distance of two cell diameters, was estimated to be ~9\%, based on the calculated potassium permeability method of Firestein and Werblin (1989). Chemical concentrations are reported thus as the pipette concentration and are not corrected for this dilution. The pipette concentration of each of the body secretions was 1:300. All dilutions were prepared fresh daily in reptile Ringer, which served as the control vehicle in all conditions. If a neuron responded to the control, it was assumed to be a mechanical stretch-activated response, and no further use of the cell was made. The peak magnitude of a response was measured as the difference in current from the baseline prior to presentation of the chemical to the peak outward- or inward-evoked current within 500 ms of valve activation of the picospritzer. Zero current (no response) was defined as no observable deflection or deflection of current less than fourfold the total noise level (membrane plus equipment) under control baseline conditions.

The number of different compounds that stimulated a given cell (the response spectrum) was quantified using the breadth of responsiveness metric of Smith and Travers (1979). Here, the breadth of responsiveness ($H$) is defined as

$$H = -K \sum_{i=1}^{N} p_i \log p_i,$$

where $K$ is a proportionality constant, $N$ is the number of chemicals tested, $p_i$ is the absolute current (pA) elicited by the $i$th chemical and expressed as a proportion of the total pA elicited by all chemicals. This equation is an application of the entropy equation as a measure of diversity in neural responsiveness. The $p_i$ for each neuron are derived by converting the neural response profile for that neuron, into a proportional profile, where the response to each chemical is expressed as a proportion of the total response to all five chemicals (Smith and Travers 1979). The value of $K = 1.4306$ was calculated so that $H = 1.0$ when $p_i = 1/N$. Therefore when there is a response to only one of the five chemicals, breadth of responsiveness is minimum ($H = 0$), and it is maximum ($H = 1.0$) when there is an equal response to each of the five chemicals (no selectivity or diversity in response) (Smith and Travers 1979).

RESULTS
Vomeronasal organ (VNO) in Liolaemus is compartmentalized from the main olfactory epithelium

Although an extensive range of literature has chronicled the chemosensory behavior of the lizard in the genera *Liolaemus* (Labra and Niemeyer 1999, 2004; Labra et al. 2001, 2003), there has been no report of the basic anatomy of the organ presumed to transduce chemical secretions into an electrical event. Nor has there been characterization of GTP-binding proteins present in the VNO that could couple to pheromone receptors in the first stage of establishing the transduction current. Thus to ascertain that we were indeed functionally characterizing the sensory neurons in the VNO, we first explored the organ using tracer dyes, histological stains, and immunocytochemical techniques. Due to the discovered arrangement that the main olfactory epithelium (MOE) and the VNO were comprised as two large, adjacent, bilaterally symmetrical lumena, it was necessary to decalcify the skull prior to cytosectioning to preserve the structure without compression (see METHODS). As shown in Fig. 1, the sensory epithelium of the VNO was distinct from that of the nonsensory epithelia. Like VNO found in other reptiles, that of *Liolaemus* was relatively large in comparison to the MOE, making the VNO amenable to functional electrophysiology (Murphy et al. 2001; Taniguchi et al. 2000). Dissimilar to that of some other reptiles, however, the two organs appeared to be completely segregated
from one another and not merely separated by a cartilaginous ridge within a single organ. To test this perception and confirm connectivity to the internal nares in the upper palate of the animal, we delivered rhodamine-conjugated dextran by means of a mini pump into the presumed VNO orifice and tracked vital dye migration over a period of two weeks. Using a similar approach that was developed for olfactory sensory neurons (Wachowiak and Cohen 1999), the dextran is also mixed with triton detergent that shears microvillar processes and allows the dextran to enter the dendritic extensions of the sensory neurons. As shown in Fig. 2, labeling was observed in the vomeronasal sensory neurons on the ventral half of the animal to which the dye was introduced. There was no apparent migration of the dye to the other half of the VNO or the adjacent, dorsal MOE, inferring the compartmentalization of the VNO. It is not known if the noted medial location of the labeled neurons is a property of dye perfusion or clustering of a distinct subclass of sensory neurons in this region; three dextran-perfused animals had a similar pattern of localization. Preliminary immunohistochemistry experiments, however, that do not require solution migration, also demonstrate a higher intensity of label against anti-G_{β} in this same medial location (data not shown).

The VNOs from non-dye-labeled animals were prepared into membrane proteins and separated by SDS-PAGE to probe with G-protein subunit-specific antisera. Although a rhodamine-conjugated dextran infused animal was doubly labeled with anti-G_{β} by immunocytochemical procedures (data not shown), which did co-localize with the rhodamine dye signal, this approach was not practical given the level of difficulty of the sectioning. It was much easier to screen many antisera by biochemical techniques and use less animals so this was the selected approach. Labeled protein of expected molecular weight was observed in both species for G_{β}, G_{α_i}, and G_{α_o} specific antisera (Fig. 3). For this biochemical survey of G proteins, only male lizards were tested to conserve the collected female lizards to balance sex ratios sampled in the later electrophysiology experiments. Due to the highly evolutionarily conserved sequences of the G-protein family, rodent cerebellum or cerebral membranes were used as a positive migration control as previously found helpful in previous new species characterizations of G proteins in the olfactory organs of turtle and lobster using these identical stocks of antisera (Fadool et al. 1995; Murphy et al. 2001). No signal was observed when the primary antisera were omitted (Fig. 3). Antisera directed against G_{α Rift}, G_{α_i}, or G_{α_o} either produced no labeled protein or labeled a protein of inappropriate molecular weight in relation to the rodent positive control (data not shown).

Two different patterns of voltage-activated currents are present in Liolaemus VN sensory neurons independent of sex or species

Whole cell recordings were made from 69 isolated single vomeronasal sensory neurons from two L. nigroviridis and 21 L. bellii. We had previously explored several protocols for dissociation of neurons to achieve acute isolation of single vomeronasal neurons (Fadool et al. 2001) and thus used an incubation in weak cysteine-activated papain known to preserve chemosignal-activated conductances in mouse and turtle VNO. The neurons had a morphology similar to that reported in other vertebrate species (Taniguchi et al. 1995; Trotier et al. 1998) and isolated VN neurons remained viable for ≥8 h after initial dissection of the organ. Only neurons with full dendritic processes (presumably containing intact microvilli) and with a resting potential of at least −50 mV were considered suitable for inclusion in the study. From previous investigations in other species, we avoided neurons that appeared not to have full dendritic processes as these types typically were unresponsive to chemicals likely due to damage during the trituration step used for isolation (Fadool et al. 2001). The mean resting potential for an arbitrary subset of monitored neurons was −59 ± 2 (SE) mV (n = 16). Neurons that had an initially
observed resting potential more positive than −50 mV were considered likely damaged during the isolation process, and thus no further use of these cells was considered. Neurons were voltage-clamped at −90 mV \((V_h)\) and then stimulated for 400 ms in 5-mV depolarizing steps to +40 mV \((V_c)\) using a 10-s interpulse duration (Fig. 4). The total membrane current evoked in a typical cell consisted of a rapid inward current \((-10 \text{ ms duration})\) that activated around −40 mV, followed by an outward current that contained both a transient and sustained component, which activated between potentials of −30 and −40 mV. For the family of traces presented in Fig. 4, A and B, this pattern of activation can be seen in the current-voltage \((I-V)\) relationships (Fig. 5, A and B).

There was a marked difference in the electrical response of these neurons to voltage-stimulation with some neurons containing a relatively equal proportion of K current compared with that of Na current and other neurons containing very little K current as opposed to a dominant Na current. We classified these two types of response profiles according to the plotted family of current voltage relationships \((I-V)\) as the “large K current” profile type (“large K,” Fig. 5A) and the “small K current” profile type (“small K,” Fig. 5B). Representative recordings taken from both \emph{L. nigroviridis} and \emph{L. bellii} demonstrate that these electrical responses were neither species- nor sex-dependent (Fig. 4, A–D). The mean peak current amplitude for the two components of the outward K current at +40 mV and the peak inward Na current at −20 mV was not statistically different when compared across sex (Table 1). The inactivation kinetics of the outward current \((\tau_{\text{inact}})\) and the deactivation kinetics of the tail current \((\tau_{\text{deact}})\) were calculated by regression analysis (see METHODS) and also statistically compared (Table 1) to show no difference across sex. These data are plotted in Fig. 5C to demonstrate that the peak current magnitude of the neurons does not exhibit any sexual dimorphism, and when neuron recordings are sorted independent of sex or species (Fig. 5D), there are two different classes of electrical profiles with quantifiable differences in the ratio of expressed Na : K current \((1.3 \pm 0.1, n = 17, \text{large K current vs. } 5.9 \pm 0.7, n = 12, \text{small K current}; \text{Student’s } t\text{-test arc-sin transformation, } \alpha \leq 0.05)\). Independent of class of \(I-V\) response type, a large fraction of the outward current \((73 \pm 7\%, n = 4\) is likely contributed by a delayed rectifier potassium channel given its sensitivity to 10 \(\mu\)M tetraethylammonium (TEA; Fig. 6). Likewise the inward current is largely contributed, if not com-

![Fig. 4](image-url)
pletely contributed, by a tetrodotoxin-sensitive sodium current (97/100 4% block, n/100 8; Fig. 6; TTX concentration /10 nM). There was no difference in TTX-sensitivity across large versus small K current I-V response type (Fig. 6, A and B). Contrary to our prediction and expectation (Fioni et al. 2003), the electrical response type (I-V profile) did not correlate to the

![Fig. 5. Characterization of the two different patterns of voltage-activated whole cell currents recorded in Liolaemus VN neurons. A and B: current/voltage relationships (I-V) were plotted from the family of currents shown in Fig. 4, A and B. A: representative large K current I-V type of voltage-gated currents (Large K I-V) are shown for a female L. nigroviridis; plotted are the inward sodium currents (Na; ■), and the two components of the outward potassium current (sustained K (■) and transient K (Œ)). B: representative “small K current” I-V type of voltage-gated currents (“small K” I-V) are shown for a male L. nigroviridis. Notation as in A. C: histogram graph of the peak outward potassium and inward sodium voltage-activated currents divided per sex of the animal (♀, female; ♂, male). Data are reported as the means ± SE with sample size of the recordings as indicated. There was no significant difference in the calculated means between male and female records (Student’s t-test, α ≤ 0.05). D: histogram graph of the peak potassium (at +40 mV) and sodium (at −20 mV) voltage-activated currents where VN neurons are separated into the “large K” I-V (■) and “small K” I-V (Œ) types. Data are reported as the means ± SE with sample size of the recordings as indicated. *, significantly different by Student’s t-test, α ≤ 0.05. Mean ± SE of the Na/sustained K ratio is reported for the sampled population directly below each type. **, significantly different by Arc-sin transformation Student’s t-test, α ≤ 0.05.

Vomeronasal (VN) neurons were voltage clamped in the whole cell configuration. Neurons were held (V_h) at −90 mV and then stepped in 5- or 10-mV increments to +40 mV (V_c) using a pulse duration of 400 ms and an interpulse interval of 10 sec. Peak transient and sustained potassium (K) currents were taken at the +40-mV pulse. Peak sodium (Na) current was measured at the −20-mV pulse. The current at half-activation (V_1/2) was calculated using a fit of the plotted tail current versus voltage to the Boltzman equation. The time constant or tau for inactivating (τ_inact) and deactivating (τ_deact) portions of the potassium current was calculated as described in the text for the +40-mV pulse. Values are reported as the means ± SE with sample size (in parentheses). Additional recordings were made for L. nigroviridis (7 male, 4 female) species with comparable results (data not shown).

**TABLE 1. Biophysical properties of vomeronasal neurons acutely isolated from male and female L. bellii**

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<th>Transient K, pA</th>
<th>Sustained K, pA</th>
<th>Na Current, pA</th>
<th>V_1/2, ms</th>
<th>τ_inact, ms</th>
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<td>345.9 ± 57.7 (25)</td>
<td>289.0 ± 66.0 (25)</td>
<td>604.8 ± 104.9 (15)</td>
<td>6.8 ± 1.4 (25)</td>
<td>142.7 ± 19.5 (21)</td>
<td>7.1 ± 1.9 (23)</td>
<td></td>
</tr>
<tr>
<td>351.9 ± 41.3 (33)</td>
<td>263.0 ± 41.8 (33)</td>
<td>626.8 ± 74.9 (15)</td>
<td>6.4 ± 1.4 (33)</td>
<td>198.1 ± 31.8 (27)</td>
<td>5.7 ± 1.0 (29)</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>349.3 ± 34.2 (58)</td>
<td>274.2 ± 37.1 (58)</td>
<td>616.2 ± 64.1 (30)</td>
<td>6.6 ± 1.0 (58)</td>
<td>174.3 ± 20.3 (48)</td>
<td>6.3 ± 1.0 (52)</td>
</tr>
</tbody>
</table>

Vomeronasal (VN) neurons were voltage clamped in the whole cell configuration. Neurons were held (V_h) at −90 mV and then stepped in 5- or 10-mV increments to +40 mV (V_c) using a pulse duration of 400 ms and an interpulse interval of 10 sec. Peak transient and sustained potassium (K) currents were taken at the +40-mV pulse. Peak sodium (Na) current was measured at the −20-mV pulse. The current at half-activation (V_1/2) was calculated using a fit of the plotted tail current versus voltage to the Boltzman equation. The time constant or tau for inactivating (τ_inact) and deactivating (τ_deact) portions of the potassium current was calculated as described in the text for the +40-mV pulse. Values are reported as the means ± SE with sample size (in parentheses). Additional recordings were made for L. nigroviridis (7 male, 4 female) species with comparable results (data not shown).
magnitude, breadth of responsiveness, or type of body secretion for chemosignal-activated conductances (following section) as measured in *L. bellii*.

**Chemically activated properties in *L. bellii* vomeronasal sensory neurons**

Forty-six sensory neurons from eight male and four female *L. bellii* were held at −60 mV (below the potential for voltage-activation) and stimulated for 700 ms with five different body secretions that were prepared as whole crude extracts (see **METHODS**) namely: male skin, female skin, male feces, female feces, and male precloacal secretion (Fig. 7A). Due to our animal collection sample from Chile, only *L. bellii* and not *L. nigroviridis* were used for study of chemosignal-activated conductances. Thus for this initial study, we did not test a secretion back on the same donor, across species nor did we test within known siblings; all secretions were harvested from reproductively active adult specimens (*L. bellii*) and applied to other adults not housed in the same terraria. If a neuron responded to control saline (Ringer), it was considered a mechanical artifact or stimulation of a stretch receptor and the neuron was discarded from the analysis. Only neurons that were stable through application of all six solutions (Ringer plus 5 body secretions) and did not demonstrate a change in series resistance were retained in the study. An example response from a typical neuron isolated from *L. bellii* (male) and stimulated with the described battery of chemosignals is shown in Fig. 7C. Current responses typically rose to a maximum over several hundred milliseconds and subsequently declined to rest over a period of 3–4 s. This pattern of current deflection was never randomly observed (absence of stimulation) and was always time-linked to stimulus presentation within 500 ms of valve activation. Although the properties of adaptation were not formally tested, a current response to a body secretion applied ten times during a 30-min recording period yielded responses that only varied by 2–5%. We did not observe a pattern of responsiveness that was sex-dependent. The VN responses from a male and female animal to male precloacal secretion demonstrates this independence in Fig. 7D. Likewise, neurons harvested from either males or females could respond to both male and female secretions. The response could be either in the form of an inward or outward current and both polarity of chemosignal-evoked currents could be observed within a single neuron. Although neurons could respond with dual polarity of chemosignal-evoked currents to different secretions, a single body secretion could not evoke both polarity within a neuron, only across a population of neurons. The inward chemosignal-evoked current was associated with a conductance increase as measured by injecting a hyperpolarizing voltage step from $V_h$ to $V_{op}$ and subsequently increased to rest (Fig. 7B). At rest the VN neurons had a mean input resistance ($R_N$) of 1.8 ± 0.4 GΩ and a membrane time constant ($\tau_m$) of 24.8 ± 6.4 ms ($n = 4$). During chemosignal stimulation, the $R_N$ decreased significantly to 1.5 ± 0.3 GΩ and the $\tau_m$ decreased significantly to 9 ± 3.3 ms (paired t-test, $\alpha = 0.05$). Although the change in $R_N$ value represents a statistical difference, it is not clear if this would provide a physiological meaningful alteration at the level of the cell membrane. The response magnitude of a chemosignal-evoked conductance had a range of −40 to +60 pA with most responses clustering in the −20 to +20-pA range (Fig. 8A). The rank order of effectiveness defined as the body secretion with the greatest frequency of response (not greatest strength of response quantified by peak magnitude) was: male skin, female feces, female skin, male precloacal secretion, and then male feces (Fig. 8B). Most secretions were capable of evoking both polarity of current response (not in the same cell) with the exception of male precloacal secretion, which only evoked outward current, and male feces that strongly elicited inward current (Figs. 8B and 7D). Although it will be important to systematically sort reversal potentials of chemical-activated currents with type of secretion, sex, and current polarity, current reversal for female feces applied to a VN neuron taken from a female animal demonstrates an estimated current reversal near +4 mV (Fig. 7E and F).

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**FIG. 6.** Pharmacological block does not distinguish between the 2 voltage-activated currents types in *Liolaemus* VN neurons. A: representative voltage-activated currents for a neuron held ($V_h$) at −90 mV and stepped in 10-mV depolarizing potentials ($V_d$) to +40 mV for 400 ms with a 10-s interpulse interval. This neuron was classified as the large K $I-V$ type, and the family of currents on the left were control traces prior to toxin application (control), and those on the right were recorded 5 min after 10 nM tetrodotoxin (TTX) bath application (TTX). B: same as in A, but for a neuron classified as the small K $I-V$ type. Voltage stimulation paradigm was the same as in A except depolarizing increment was 5 mV instead of 10 mV. C: same voltage paradigm as in B. This neuron was classified as the large K $I-V$ type and was blocked for 15 min with 10 nM tetrodotoxin to eliminate any Na channel contribution to the control trace (control) as compared with that after 10 μM tetraethylammonium (TEA). Similar magnitude of TEA block was found for neurons of the small K $I-V$ type (data not shown).
**Breadth of tuning of chemosignal responses in the vomeronasal sensory neurons**

Of the forty-six neurons tested with all five body secretions, 59% (n = 27) elicited a current response to at least one of five test stimuli. Of the 27 responsive cells, 11 cells responded to only one of the five stimuli presented in the array. The other responsive cells (16 of 27 cells) responded to two or more stimuli (Fig. 9). Thus the cells varied in the number of chemicals to which they responded and the magnitude and polarity of the response to a given stimulus. The frequency of the breadth of responsiveness (H) for this sample population is plotted in Fig. 9B (H = 0.29). Note that the magnitude of the responses also enters into the calculation for entropy. Because this mean H metric contains a subpopulation of VN neurons that are most selective to a single chemical (H = 0) and a subpopulation that is more broadly tuned (H > 0.3), the plotted histogram distribution provides a better representation in that there may be a heterogeneous distribution of tuning for a given VN to a battery of chemosignals.

**DISCUSSION**

Our study represents the first anatomical description of *Liolaemus* VNO structure and the first characterization of G-protein subunit expression in the VNO of any lizard species. The discovered high chemosensitivity of the VSNs in lizard (59%) can now facilitate a detailed analysis of chemical communication at the level of the ion channel. Previous electrophysiological studies predominantly focused on population coding or multiunit patterns of activity given the lower response rate to pheromones in rodent VNO (Holy et al. 2000; Leinders-Zufall 2000). Important questions concerning transduced information and level of electrical integration at single neurons can now be explored using lizard.

A good comprehensive review of the earliest descriptions of reptilian nasal anatomy can be found in Parsons (1958). Much of the early research as well as currently re-visited lizard VNO anatomical studies were concerned with the chemical access of the organ and its mode of lubrication, rather than its neurosensory capacity or structure (Graves 1993; Graves and Halpern 1989; Rehorek et al. 2000). Light microscopy of the VNO of *L. bellii* shows similar anatomical features as those described for the blue-tongued lizard and ocellated skink (Graves and Halpern 1989; Kratzing 1975). One notable exception is that the cross-sectional area of the VNO in the *Liolaemus* lizard approaches that of the MOE, whereas in the other two lizard species, the VNO is only 1/3 the cross-sectional size of the larger MOE. The two partitions of the olfactory system, MOE and VNO, are easily discernable in all three lizard species (Graves and Halpern 1989; Kratzing 1975; this study) with the noted presence of the mushroom body and the absence of Bowman’s glands in the VNO.

The first expression of GTP-binding proteins within the VNO was reported for another reptile, the garter snake (Luo et al. 1994). Across animal phyla, including mammalian, amphibian, and reptilian members, three G-protein subunits are consistently expressed in the VNO (G_	ext{o}, G_	ext{alt}, and G_	ext{q}) (Berghard and Buck 1996; Jia and Halpern 1996; Luo et al. 1994; Murphy et al. 2001; Wekesa and Anholt 1997). Our data are complementary to these reports and also demonstrate the lack of protein labeling for G_	ext{o}, G_	ext{alt}, and G_	ext{q}, which are reported in the main olfactory epithelium of many species but absent in the VNO. From an evolutionary perspective, some reptiles have two segregated transduction epithelia (Luo et al. 1994) similar to that reported for rodents and others do not (Murphy et al. 2001). Future immunocytochemical and in situ approaches will be needed to determine degree of zonal segregation, if any, in the lizard.

The voltage-activated sodium and potassium currents expressed in the VSNs appear to be heterogeneous yet simultaneously dependent of *Liolaemus* species or sex. The ratio of sodium to potassium current magnitude was six in one population of VSNs and close to unity in a second population of VSNs. Although a proportion of the difference in sodium to potassium current ratio was attributable to a twofold increase in sodium current, a very marked difference in the TEA-sensitive potassium current also existed across the two classes of VSNs, where in some neurons, the corresponding K current could be as little as 50–100 pA. K channels classically govern the width of the action potential, define the duration and magnitude of the after hyperpolarization and drive the timing of the interpulse interval (Yi et al. 2001). Therefore such a reduction in K channel contribution would strongly predict an increase in neuronal excitability in the “low K” class of VSNs. Fieni et al. (2003) interestingly report differential biophysical properties of apical versus basal vomeronasal sensory neurons in the mouse. These authors report an increased sodium channel density in the apically located VSNs as opposed to those located in the basal portion of the epithelium. Although our experimental design using acutely isolated neurons, as opposed to the slice configuration, does not preserve position information, it is not inconceivable that our two classes of neurons might correspond to segregated neurons within the epithelium, especially given that we found no correlation to sex or species. A future important alternative approach would be to postlabel recorded neurons with either anti-V1R or -V2R to determine if electrical phenotype correlated to transduction pathway. Second, it is interesting that in animals that lack segregation of transductionary cascades, there still exists heterogeneity in the current magnitude of sodium current. For example, in turtles, VSNs from males have roughly twice the sodium current as that expressed in VSNs from females even though the entire population of VSN only expresses a single type of G_	ext{iat} protein (Murphy et al. 2001). Whether more than one neuronal category exists in the VNO as defined by sex, epithelia position, or another factor, the commonality across organisms (mouse, turtle, lizard) appears to be that of a change in channel density, which can influence the neuronal excitability and ultimately the generation of action potentials. This has important implications for the coding of chemosignals that are thus highly dependent on functional membrane properties across individual neurons.

Vertebrate animals perform patterned behaviors that may be involved in either investigation of the chemosignal or promoting access of the chemosignal to the VNO (Wyatt 2003). *L. bellii* increases its frequency of tongue-flicking during the reproductive period (Labra et al. 2001, 2003), and in general, *Liolaemus* lizards increase flicking in response to substrates with novel social pheromones (Labra and Niemeyer 1999, 2004; Labra et al. 2001, 2002). In the present study, it is interesting to note that male skin is the body secretion that...
produced the highest frequency of response. A possibility that cannot be excluded from our data, is that homogenization of the skin could have released substances that could stimulate the VN neurons, which are not normally accessible on the surface of the skin behaviorally. At the present, there are no behavioral studies in *Liolaemus* that test the role of skin as a pheromone.
production site, although this has been reported in other lizard species (Bull et al. 2000; Mason and Gutzke 1990). In addition, the function of the precloacal secretions has been ignored for many years. Escobar et al. (2001), following the propositions of Donoso-Burros (1966), suggested pheromonal properties of precloacal secretions; our data demonstrate that VN neurons respond electrically to precloacal secretions, both by males and females during the reproductive season.

Although it was not possible to present whole crude secretions to individual VN sensory neurons without clogging our multibarrel odor delivery pipette, our clarification of the secretions was minimal using only low-speed centrifugation. Purified single pheromones have been shown to evoke electrical signals in mammals (Leinders-Zufall et al. 2000) and certainly chemical modification of single compounds can lessened responsivity, nonetheless we wanted to mimic the natural scenario by presenting the entire pheromone-containing body secretion to determine the electrophysiological response. The high rate of chemosignal-evoked responses (59%) in individual VN sensory neurons surpassed that of mammals (individual pheromones 2–3%; dilute urine 38%), where only optical sensors exhibited inward outward current responses was minimal using only low-speed centrifugation. Puriﬁcations to individual VN sensory neurons without clogging our multibarrel pipette in series with a pressurized valve system for a duration of 700 ms (bar). Dashed line, basal current prior to valve activation.

The wider breadth recorded in Liolaemus may determine the possibility that their VNO can detect a wider range of chemosignals. Alternatively, we do not know if the high response rate in the lizard VN sensory neurons is a reflection of our lack of puriﬁcation of the stimulus solution or an innate property of these neurons. An additional mitigating variable is selection of multibarrel pipette in series with a pressurized valve system for a duration of 700 ms (bar). Dashed line, basal current prior to valve activation.

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are periodically triturated over the recording session, it is not certain whether mucous or mucal components from the intact quarters are carried into the recording dish. Nonetheless, our data indicate that the VN neurons are selectively excitable and selectively inhibited to a defined breadth of secretions. The VN neurons tested for chemically evoked currents did not all respond identically to the same compound as is classically observed in neurons responding to other chemicals, such as neurotransmitters or neuromodulators. A homogeneity of response might also be expected if the neurons were responding to a uniform concentration of potassium ion excreted in the secretion. Even though the diversity of response profiles (Fig. 9) was not consistent with this possibility, we tested boiled secretions or secretions that had been subjected to repeated freeze-thaw cycles, both of which failed to produce a response. Interestingly, although not formally quantified, we discovered that there was a decrease in response rate after the reproductive period of the animal, implying that either the composition of the secretion, the production of the transduction machinery, or both were altered as the season transitioned to typical hibernation of the species. These data are consistent with seasonal changes in intraspecific chemical recognition reported in  *Liolaemus*, which have been shown to decrease their chemical exploratory behavior (tongue flicking) in the postreproductive season (Labra and Niemeyer 1999).

Although inhibitory conductances have not been reported in rodent VNO preparations, it is noteworthy that outward chemosignal-evoked currents are reported in reptiles (musk turtle, this study). In the lizard VNO, both polarity of chemosignal-evoked currents were observed with a single VSN. Precloacal secretions, in fact, only were observed to evoke outward currents in both male and female VSNs. It cannot be discerned if outward currents (presumably inhibitory) are particular to this species, but certainly inhibitory odor-evoked responses are observed for olfactory sensory neurons (OSNs) in both invertebrate and vertebrate species alike (Delay and Restrepo 2004; Michel and Ache 1994; Pun and Kleene 2002; Sanhueza et al. 2000), yet have been as a whole, poorly studied. It thus may be a matter of sampling frequency or identified inhibitory chemosignal/odorant.

This study represents the initial characterization of a sensory organ in an animal model that will afford a tactical advantage for single-cell electrophysiology in the VNO. The high rate of chemosignal-evoked responses (59%) in individual VN sensory neurons tied with the heterogeneity of voltage-dependent properties makes future studies of pheromone transduction both practical and intriguing. The rich chemosensory environment and behavioral displays of the lizard during chemosensory discrimination will give an added dimension to future biophysical studies of lizard VNO.
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