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

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Abstract

Liolaemus lizards were explored to ascertain whether they would make an amenable model to study single-cell electrophysiology of neurons in the vomeronasal organ (VNO). Despite a rich array of chemosensory-related behaviors chronicled for this genus, no anatomical or functional data exist for the VNO, the organ mediating these types of behaviors. Two *Liolaemus* species (*L. bellii* and *L. nigroviridis*) were collected in Central Chile in the Farellones Mountains and transported to the United States. Lizards were subjected to hypothermia and then a lethal injection of sodium pentobarbital prior to all experiments described in the following text. Retrograde dye perfusion combined with histological techniques demonstrated a compartmentalization of the proportionally large VNO from the main olfactory epithelium (MOE) in cryosections of *L. bellii*. SDS-PAGE analysis of the VNO of both species demonstrated the expression of three G protein subunits, namely, $G_{\alpha o}$, $G_{\alpha i2}$, and G_{β} , and the absence of $G_{\alpha olf}$, $G_{\alpha 11}$, and G_q , the latter of which are traditionally found in the MOE. Vomeronasal (VN) neurons were enzymatically isolated for whole cell voltage-clamp electrophysiology of single neurons. Both species demonstrated a tetrodotoxin (TTX)-sensitive, rapidly inactivating sodium current and a tetraethylammonium (TEA)-sensitive potassium current that had a transient and sustained component. VN neurons were classified into two types dependent on the ratio of sodium over sustained potassium current. VN neurons exhibited outward and inward chemosignal-evoked currents when stimulated with pheromone-containing secretions taken from the feces, skin, and precloacal pores. Fifty-nine percent of the neurons were responsive to at least one compound when presented with a battery of five different secretions. The breadth of responsiveness (H metric) demonstrated a heterogeneous population of tuning with a mean of 0.29.

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 Martínex-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; Leypold 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, $G_{\alpha o}$ and $G_{\alpha i2}$, 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 Martínez-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; 243 mOsm). Ca²⁺-free Ringer used for neuron isolation contained (in mM) 116 NaCl, 4 KCl, 1 MgCl₂, 15 glucose, and 5 NaPyruvate (pH 7.4). Phosphate-buffered saline (PBS) contained (in mM) 136.9 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 Na₃VO₄. 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 Na₃C₆H₅O₇ · 2H₂O, 10 β-mercaptoethanol, with 1% SDS; pH 3.0.

The rabbit polyclonal antisera anti-G_β (1:950) and anti-G_{α1-3} (1:500) were purchased from Santa Cruz (Santa Cruz, CA). Anti-G_β was raised in rabbits immunized with a synthetic decapeptide MSELDA LRQE (amino acids 1–10 of bovine transducin β subunit). Anti-G_{α1-3} was raised against a peptide corresponding to an amino acid sequence within a highly divergent domain of G_{α1} so that it would react with G_{α1-3}. Mouse monoclonal anti-G_{αo} (1:10,000) was purchased from BD Biosciences (San Jose, CA) and was generated against amino acids 161–171 of the human form of G_{αo}. Anti-G_{αolf} (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 KTAEDQGVDEKERREA, near the amino terminus of rat G_{αolf}) (Jones and Reed 1989). Anti-G_{αq} (E973), made to amino acids 115–133 of the G_{αq} peptide sequence and anti-G_{α11} (E976), made to amino acids 160–172 of the G_{α11} sequence (Strathmann and Simon 1990), were gifts from Dr. John Exton (Vanderbilt University, Nashville, TN). Affinity-purified anti-G_{αq/11}, a sequence conserved across species from human to snail (an amino terminal cysteine coupled to the last 12 amino acids: CKLQLNLKEYNLV) (Gutowski et al. 1991) was a gift from Dr. Paul Stern-weiss (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 μ l 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 μ l 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 reintroduction 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% paraformaldehyde in PBS for 3 h followed by overnight infiltration with 10% sucrose in PBS, then 4 h with 30% sucrose in PBS, all at 4°C. After cryoprotection by sucrose infiltration, the lizard skull was decalcified by room temperature incubation in 0.3M EDTA in PBS for 48 h on a rotary platform (approximately 500 RPM). Heads were then cut to 9- to 12- μ m thickness on an Ames Microtome-Cryostat (Model No. 4550; Tarrytown, NY). Sections were transferred to 1% gelatin-coated glass slides (Sigma) and stored at –20°C until use.

Histochemistry and photomicroscopy

Rhodamine-conjugated dextran labeled cryosections were viewed at $\times 10$ and $\times 40$ magnification without any further treatment using an Axioplan Zeiss Microscope equipped with epifluorescence and photographed using a Zeiss AxioCam digital camera. Other nonlabeled cryosections were immunochemically labeled using permeabilization of the tissue with 0.1% Triton X-100 and protocols as previously described (Murphy et al. 2001). Nuclear stain was achieved by inclusion of 4,6-diamidino-2-phenylindole (DAPI) (1:50,000) (Sigma) in the second to last wash using PBS plus 0.1% Triton X-100 prior to mounting. The digital

images of each section were captured with Zeiss AxioVision v3.0.6 software at a pixel resolution of $1,300 \times 1,300$. Digital files were imported into Photoshop (Adobe 5.5) to facilitate multiple panel/figure output.

Protein chemistry

Male lizards were anesthetized by chilling at 4°C and then killed by administering a lethal injection of sodium pentobarbital (Butler) followed by decapitation. Adult Sprague Dawley rats were killed by carbon dioxide inhalation and then killed by decapitation as per NIH- and AVMA-approved methods as governed by the ACUC at FSU. VNOs (lizard) and cerebellum or cerebral hemisphere (rat) were quickly dissected and homogenized on ice for 50 strokes in HB + PI additions with a Kontes tissue grinder (size 20). Tissue homogenates were centrifuged twice at 15,800g (Eppendorf 5415, Hamburg, Germany) for 30 min each at 4°C to remove cellular debris. Supernatants were then centrifuged at 110,000g for 1.5 h at 4°C (Beckman TLA 100.3, Fullerton, CA). The resultant pellet was resuspended by sonication (setting 10, output power 3 watts, 3×3 s pulses, Model 60 Sonic Dismembrator, Fisher Scientific), assayed for protein concentration by Bradford Assay, and stored at -80°C until use.

Membrane proteins (15 µg/lane) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels using electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose membranes. Blots were blocked with 5% nonfat milk for 1 h and then incubated overnight at 4°C in subunit-specific G-protein primary antisera. Proteins were visualized by peroxidase-conjugated species-specific secondary antisera incubation followed by enhanced chemiluminescence (ECL; Amersham-Pharmacia, Piscataway, NH) on Kodak X-Omat AR-5 film (Fisher Scientific, Suwanee, GA). Where necessary, blots were stripped in rotary vials at 46°C (Isotemp Oven, No. 13×247×10, Fisher Scientific) using eight 10-min washes in TSB followed by eight 10-min washes in SCSB. Blots were then blocked and reprobed as in the preceding text. Resultant autoradiographs were stored using a Hewlett-Packard Photosmart Scanner.

Electrophysiology

Liolaemus lizards were anesthetized and killed as in the preceding text. Neurons from the VNO were isolated by incubation in Ca^{2+} -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. 25000) (Leinders-Zufall et al. 1997). Isolated vomeronasal neurons were viewed at $\times 40$ magnification (Axiovert 135, Carl Zeiss, Thorn-wood, 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, Bedford-shire, 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 (V_h) 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 pClamp8.0/9.0 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 outward 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 ($y = y_0 + A_1 e^{-(x-x_0)/\tau_1} + A_2 e^{-(x-x_0)/\tau_2}$). The two inactivation time constants (τ_1 and τ_2) were combined by multiplying each by its weight (A) and summing. The deactivation of the macroscopic current was fit similarly but to a single exponential ($y = y_0 + A e^{-(x-x_0)/\tau}$). Differences between sexes in a particular biophysical property were analyzed by $F_{\max}$ test and then by Student's t -test. No tests with unequal sample sizes violated homogeneity of variance (i.e., none failed the $F_{\max}$ test) and statistical significance in all tests was defined at the 95% confidence interval (Steel and Torrie 1980).

Chemosignal 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, $p_i = |pA|$ the absolute current (pA) elicited by the n 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

Vomer nasal 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 lumina, 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 dextranperfused 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 doubled 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} , $G_{\alpha i}$, and $G_{\alpha o}$ 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 $G_{\alpha olf}$, $G_{\alpha 11}$, or $G_{\alpha q}$ 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 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 -40 and -30 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. 5*A*) and the “small K current” profile type (“small K,” Fig. 5*B*). Representative recordings taken from both *L. nigroviridis* and *L. bellii* demonstrate that these electrical responses were neither species- or 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 (τ_{inact}) and the deactivation kinetics of the tail current (τ_{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. 5*C* 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. 5*D*), there are two different classes of electrical profiles with quantifiable differences in the ratio of expressed Na : K current (1.3 ± 0.1 , $n = 17$, large K current vs. 5.9 ± 0.7 , $n = 12$, small K current; Student's *t*-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 $10 \mu\text{M}$ tetraethylammonium (TEA; Fig. 6). Likewise the inward current is largely contributed, if not completely contributed, by a tetrodotoxin-sensitive sodium current ($97 \pm 4\%$ block, $n = 8$; 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 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 (-60 mV) to a V_c of -90 mV prior, during, and subsequent to odor (body secretion) stimulation (Fig. 7B). At rest the VN neurons had a mean input resistance (R_N) of 1.8 ± 0.4 G Ω and a membrane time constant (τ_o) of 24.8 ± 6.4 ms ($n = 4$). During chemosignal stimulation, the R_N decreased significantly to 1.5 ± 0.3 G Ω and the τ_o decreased significantly to 9 ± 3.3 ms (paired *t*-test, $\alpha \leq 0.05$). 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. 7, E and F).

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 ($G_{\alpha o}$, $G_{\alpha i2}$, and G_{β}) (Berghard and Buck 1996; Jia and Halpern 1996; Luo et al. 1994; Murphy et al. 2001; Wekesa and Anholt 1997). Our data are complimentary to these reports and also demonstrate the lack of protein labeling for $G_{\alpha olf}$, $G_{\alpha 11}$, and $G_{\alpha q}$, 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 cascades (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 independent 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 transducing 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 $G_{i\alpha 1-3}$ 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-Barros (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 analysis or multi-array recording of a population of neurons is practical (Holy et al. 2000; Leinders-Zufall et al. 2000). The diversity of responses

also demonstrates that the lizard VN neurons exhibited a degree of selectivity (mean H metric value: 0.29) when presented with an array of five different compounds. The H metric can be compared with that of olfactory neurons ($H = 0.2\text{--}0.35$) and gustatory neurons ($H = 0.546$), which show a slightly greater mean breadth of responsiveness or tuning (Derby et al. 1984; Fadool et al. 1993; Smith and Travers 1979). It is interesting that the only other H metric calculated for VN neurons ($H = 0.11$) is much more narrowly tuned (Fadool et al. 2001).

The wider breadth recorded in *Liolaemus* may determine the possibility that their VNO can detect a wider range of chemo-signals. 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 electrophysiological configuration. The VNO slice preparation applied to rodent not only retains normal synaptic interactions but potentially preserves mucous and associated proteins that might be important for pheromone binding and regulation of the GPCRs. Because our preparation uses VNO quarters that 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 chemo-signal/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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FIG. 1.

Light microscopic visualization of the lizard (*Liolaemus bellii*) vomeronasal organ. *A* and *B*: bright-field photomicrograph of a 10- μ m coronal cryosection of the ventrally located vomeronasal organ (VNO) and dorsally located main olfactory epithelium (MOE); $\times 10$ and $\times 40$ magnification, respectively. - - -, midline of the organ. $\square$, the field of view enlarged in bottom panels (*B*, *D*, and *F*). Scale bar = 200 μ m (*A*, *C*, and *E*); 50 μ m (*B*, *D*, and *F*). *C* and *D*: same sections but stained with DAPI (see METHODS) to visualize nuclei throughout the organ. *E* and *F*: merged brightfield/DAPI overlay. MB, mushroom body; L, lumen; M, microvillar layer; S, supporting cell layer; VNE, vomeronasal epithelium.

FIG. 2.

Compartmentalization of the VNO and MOE in the lizard (*Liolaemus bellii*). Rhodamine-conjugated dextran inserted into the right internal naris was allowed to migrate for 2 wk. Note unilateral localization of the vital dye to the ventrally located VNO (arrow). Notation, orientation, and sectioning as in Fig. 1. Scale bar = 200 μ m.

FIG. 3.

GTP-binding proteins are expressed in *Liolaemus* lizards VNO, determined by SDS-PAGE followed by Western blot analysis. *A*: rat = control rat cerebellum membrane, ♂; = male *L. bellii* VNO membrane. *B*: rat = control rat cerebral hemisphere membrane, ♂; = male *L. nigroviridis* VNO membrane. Expected MW (in kDa) G_{β} = 30, $G_{\alpha i}$ = 33, and $G_{\alpha o}$ = 35.

FIG. 4.

Representative voltage-activated whole cell recordings from *Liolaemus* lizards vomeronasal (VN) neurons. *A–D*: sex and species as noted. Eight single, isolated VN neurons were held (V_h) at -90 mV and stepped in 5 -mV depolarizing command potentials (V_c) to $+40$ mV for 400 ms with a 10 -s interpulse interval. Two different patterns of voltage-activated currents were observed, independently of sex or species. *Inset*: faster time scale resolution for the rapidly inactivating inward (Na) current.

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 (▲)]. *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 $\pm$ 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, $\alpha \leq 0.05$). *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* (▨) types. Data are reported as the means $\pm$ SE with sample size of the recordings as indicated. *, significantly different by Student's *t*-test, $\alpha \leq 0.05$. Mean $\pm$ 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, $\alpha \leq 0.05$.

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_c) 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 μ M tetraethylammonium (TEA). Similar magnitude of TEA block was found for neurons of the small K *I-V* type (data not shown).

FIG. 7.

Vomeranasal (VN) neurons are chemically activated by different pheromone-containing body extracts or secretions. *A*: *Liolaemus bellii* cloaca (arrow); the male cloaca is on the right (asterisk). Notice the small, swollen opening or slit, which corresponds to the precloacal pores in the male. *B*: VN neuron from a female *L. bellii* was held at -60 mV (V_h) with 5 hyperpolarizing steps to -90 mV (V_c) injected prior (black), during (gray), and after (black) odor stimulation. The inward chemosignal-evoked current in this example demonstrated a conductance increase in response to male feces. Outward chemosignal-evoked currents were not tested. *C*: representative chemosignal-evoked currents from a single VN neuron from a male *L. bellii* stimulated sequentially with 5 body secretions and control Ringer. The neuron was voltage-clamped at -60 mV (V_h) in the whole cell configuration and secretions were applied through a multibarrel pipette in series with a pressurized valve system for a duration of 700 ms (bar). Dashed line, basal current prior to valve activation. *D*: representative chemosignal-evoked current elicited by a VN neuron from a male (top) and female (bottom) *L. bellii* in response to a 700-ms (bar) stimulation with male precloacal secretion. Recording configuration as in *C* with V_h as indicated. Dashed line, basal current prior to valve activation. *E*: representative chemosignal-evoked current elicited by a VN neuron from a female at various V_h as indicated when stimulated with female feces (bar). *F*: current-voltage (I - V) relation to the data in *E* is fit with a linear regression, approximating the reversal potential (E_{rev}) as $+4$ mV.

FIG. 8.

VN neurons elicit both inward and outward currents in response to chemical stimulation. **A:** histogram distribution of the peak current magnitude in response to exposure to 5 natural stimuli. The most frequent response magnitudes were between -20 and $+20$ pA. Data were binned in 2.5-pA intervals and fitted with a Gaussian distribution (—) to approximate the most frequent peak current magnitude. Zero currents were not plotted. **B:** plot of the percentage of VN neurons that respond to stimuli with inward and outward currents. The number of neurons tested is given in parenthesis on the ordinate. Notice that precloacal secretions only elicited outward currents. F, female; M, male.

FIG. 9.

Breadth of responsiveness of *L. bellii* VN neurons. **A**: representative current profiles from 10 VN neurons; magnitude of bar = strength of response as indicated by scale bar; upward bar = outward chemosignal-evoked current; downward bar = inward chemosignal-evoked current. Calculated H metric value for each neuron as indicated (see text for entropy metric explanation). **B**: histogram distribution of the breadth of responsiveness (H metric) values for a population of 27 neurons responsive to at least 1 of 5 presented chemosignals/secretions. Arrow = mean $\pm$ SE.

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

Transient K, pA	Sustained K, pA	Na Current, pA	$V_{1/2}$, mV	τ_{inact} , ms	τ_{Deact} , ms
Female					
345.9 ± 57.7 (25)	289.0 ± 66.0 (25)	604.8 ± 104.9 (15)	6.8 ± 1.4 (25)	142.7 ± 19.5 (21)	7.1 ± 1.9 (23)
Male					
351.9 ± 41.3 (33)	263.0 ± 41.8 (33)	626.8 ± 74.9 (15)	6.4 ± 1.4 (33)	198.1 ± 31.8 (27)	5.7 ± 1.0 (29)
Average					
349.3 ± 34.2 (58)	274.2 ± 37.1 (58)	616.2 ± 64.1 (30)	6.6 ± 1.0 (58)	174.3 ± 20.3 (48)	6.3 ± 1.0 (52)

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 $\pm$ SE with sample size (in parentheses). Additional recordings were made for *L. nigroviridis* (7 male, 4 female) species with comparable results (data not shown).