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Electrophysiological and Biochemical Responses of Mouse Vomeronasal Receptor Cells to Urine-derived Compounds: Possible Mechanism of Action

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Abstract

Receptor cells of the vomeronasal organ (VNO) are thought to detect pheromone-like molecules important for reproductive physiology. Several compounds derived from male mouse urine have been demonstrated to affect endocrine events in female mice. In the present study, the ability of these compounds to affect VNO activity was tested. In dissociated VNO cells held under voltage clamp conditions, application of dehydro-*exo*-brevicomin (DHB) evoked an outward current at negative holding potentials and an inward current at positive holding potentials. Under current clamp, DHB reduced action potential firing. Since DHB application caused a decrease in membrane conductance, this compound appeared to act by reducing inward current through closing an ion channel. Biochemical experiments tested the effects of DHB and 2-(*sec*-butyl)-4,5-dihydrothiazole (SBT) on cAMP levels in the VNO. A mixture of DHB and SBT decreased cAMP levels in VNO sensory tissue and had no effect on VNO non-sensory tissue. The results suggest that pheromones have an inhibitory influence on action potential generation and on cAMP levels in receptor cells of the VNO.

Over the last 15 years, substantial gains have been made in our knowledge of the morphological (Graziadei and Tucker, 1970; Hatanaka *et al.*, 1982), physiological (Keverne, 1983; Halpern, 1987; Wysocki and Meredith, 1987; Tucker, 1971) and neuroanatomical (Barber *et al.*, 1978; Itaya, 1987) features of the vomeronasal organ (VNO). More recently, isolated VNO receptor cells have been characterized with respect to electrophysiological properties (Trotier *et al.*, 1993, 1994; Taniguchi *et al.*, 1995, 1996; Liman and Corey, 1996; Trotier and Doving, 1996; Inamura *et al.*, 1997; Moss *et al.*, 1997). Some of the electrophysiological studies have indicated that VNO receptor neurons possess signal transduction mechanisms that are different from those of receptor neurons in the main olfactory system. For example, in the main olfactory system, opening of a cyclic-nucleotide-gated channel permitting entry of non-specific cations is an important step in signal transduction (Nakamura and Gold, 1987; Brunet *et al.*, 1994). However, intracellular application of cAMP to VNO receptor neurons from the frog failed to elicit a membrane current (Trotier *et al.*, 1994). Likewise, in mouse VNO receptor cells, no response to cyclic nucleotides could be detected (Liman and Corey, 1996). Additionally, repetitive firing with no sign of adaptation could be induced in VNO neurons in response to small current injections (Liman and Corey, 1996; Moss *et al.*, 1997), whereas olfactory receptor neurons fired only one or a few action potentials (Liman and Corey, 1996) or required higher current levels (Leinders-Zufall *et al.*, 1995). These data suggest that VNO receptor neurons may be more sensitive and/or more suited to detecting small signals than neurons of the main olfactory epithelium. Although the transduction processes in main olfactory neurons and neurons of the VNO appear to be different, the exact steps involved in the VNO remain unknown.

Critical to the elucidation of signal transduction in the VNO is a chemosensory stimulus which activates the VN system in a physiologically relevant manner, i.e. it alters endocrine function. Reproductive events dependent on the VNO include suppression of the estrous cycle induced by crowded housing conditions (Reynolds and Keverne, 1979), pregnancy block (Bellringer *et al.*, 1980; Rajendren and Dominic, 1984), light-induced reflex ovulation in persistent estrous rats (Johns *et al.*, 1978), full expression of sexual receptivity (Saito and Moltz, 1986; Rajendren *et al.*, 1990) and mating-induced luteinizing hormone release (Rajendren *et al.*, 1990). Two substances isolated from the urine of the adult male mouse, namely dehydro-*exo*-brevicomin (DHB)

and 2-(*sec*-butyl)-4,5-dihydrothiazole (SBT) (Wiesler *et al.*, 1984), have been shown to overcome the suppression of estrous cyclicity in overcrowded females (Jemiolo *et al.*, 1986). These two urine-derived compounds also induce aggression in males (Novotny *et al.*, 1985) and act as attractants to females (Jemiolo *et al.*, 1985). Puberty acceleration in the female mouse exposed to male urine can be blocked by removal of the VNO (Kaneko *et al.*, 1980). Recently, a group of proteins from adult male urine, the major urinary protein (MUP) complex, was demonstrated to advance puberty in the female mouse (Mucignat-Caretta *et al.*, 1995). DHB and SBT are bound to MUP (Bacchini *et al.*, 1992) as they are excreted in urine. Since DHB and SBT are weakly volatile, they may reach the VNO still bound to the protein. Cellular activation in the accessory olfactory bulb (AOB) as measured by *c-fos* mRNA expression was shown to be increased in female mice following application of a mixture of DHB, SBT and MUP to the oronasal groove (Guo *et al.*, 1997). Thus, compounds derived from male urine appear to activate the VN system to influence endocrine function.

Do urine-derived compounds activate VNO neurons?

In our experiments, the effects of several urine-derived compounds on cell membrane properties were tested in whole-cell voltage-clamp recordings. VNO neurons were dissociated by a combination of enzymatic and mechanical action (Moss *et al.*, 1997). Dissociated VNO neurons were typically of small diameter, oval in shape and bipolar. As seen in the electron micrograph in Figure 1, VNO neurons possessed an extended dendrite with a swollen knob at the distal end. Hair-like structures protruding from the swollen knob appear to be microvilli. As in the main olfactory system, it is thought that signal detection and transduction occurs at the dendritic knob. As previously reported (Moss *et al.*, 1997), a series of depolarizing and hyperpolarizing voltage steps elicited transient inward currents followed by sustained outward currents. The inward currents were sensitive to TTX while the outward currents were sensitive to TEA. The resting membrane potential, measured under current-clamp conditions, varied from –35 to –75 mV.

An outward current was observed following application of DHB to the dendritic knob of VNO neurons under whole cell patch-clamp recording. As shown in Figure 2A, DHB evoked an outward current at negative holding potentials which reversed near 0 mV. Increasing the concentration of the DHB from picomolar to millimolar levels resulted in an increase in the amplitude of the ligand-induced outward current (Figure 2B; 1 ppm = 6.5 μM; lower concentrations not shown). The dose–response curve in Figure 3 indicates that responses were detectable with concentrations in the nanomolar range and the response appeared to saturate at millimolar concentrations. The EC_{50} for DHB was determined to be 53 nm. The general odorant, *n*-amylacetate and the bath control did not evoke a response (data not shown).

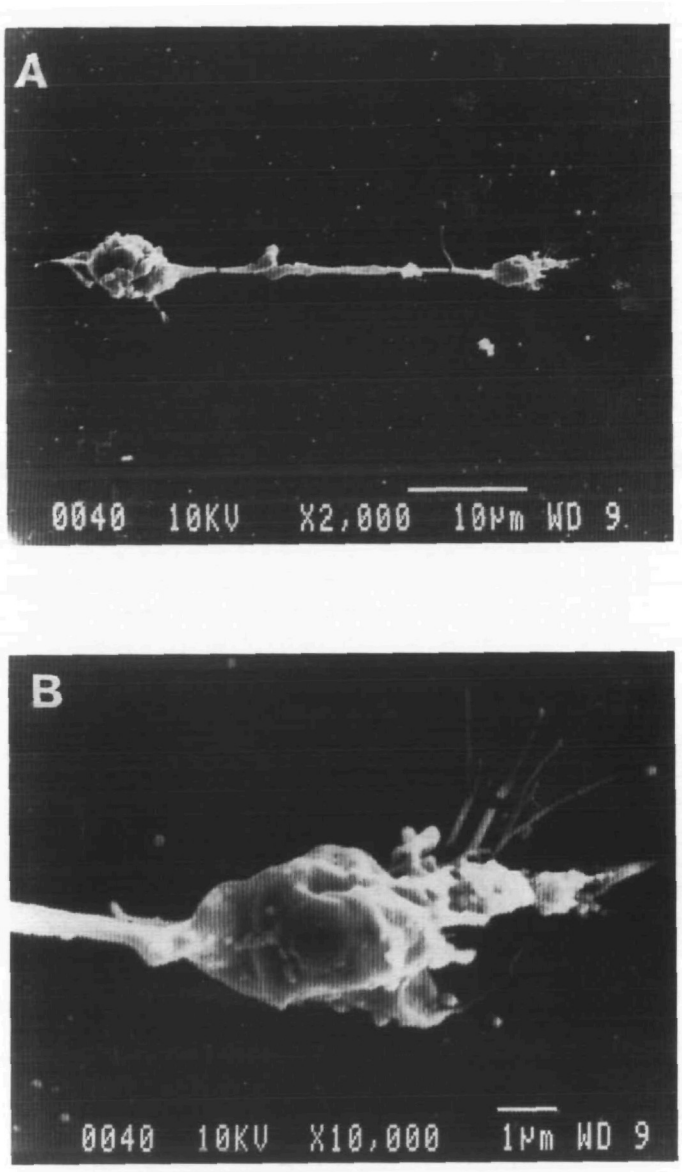

Figure 1 **(A)** Scanning electron micrograph of a dissociated VNO receptor cell. Note the broken axon to the left, the spherically shaped cell body and long dendrite. Hair-like projections extend from the dendritic knob. **(B)** Dendritic knob under higher magnification. VNOs were removed and placed in oxygenated low Ca^{2+} saline solution with subtilopeptidase BPN′ (1.5 mg/ml) and 0.2 M urea at 22°C for ~45–60 min. Following dissociation, the bipolar neurons were visualized with Nomarksi optics on an inverted microscope while in oxygenated bath solution (in mM): NaCl, 110; KCl, 2.0; $CaCl_2$, 4.0; D-glucose, 3.0; HEPES 5.0; pH = 7.25. This bath solution was used throughout the electrophysiological experiments.

Under current-clamp conditions, application of as little as 1 pA elicited repetitive firing of action potentials. In Figure 4, the VNO neuron fired repetitively in response to the application of a 2 pA current. Addition of DHB inhibited the action potential firing evoked by current application. Occasionally, as in Figure 4, DHB also induced membrane hyperpolarization.

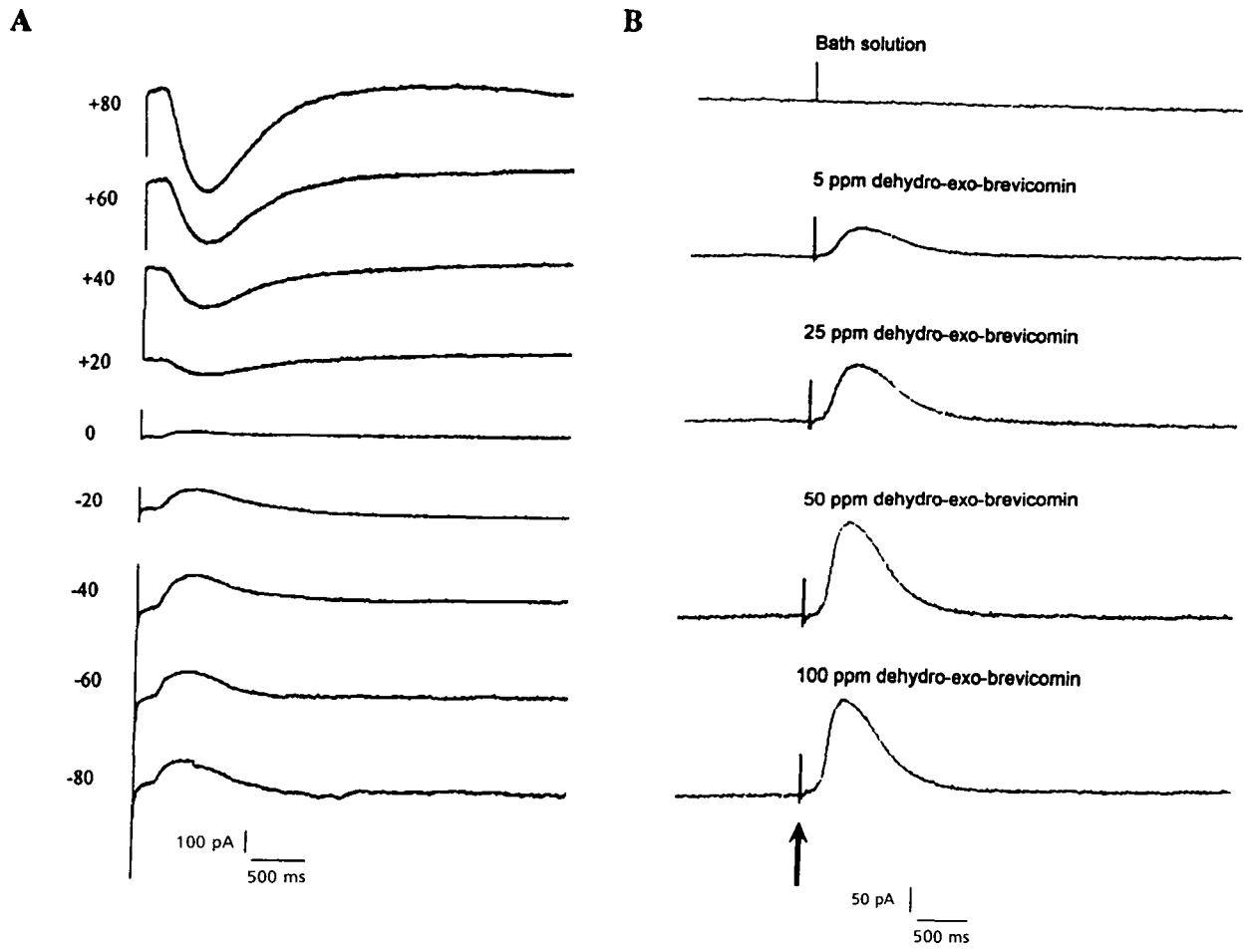

Figure 2 Responses of VNO neurons to DHB puffed onto the dendritic knob. **(A)** This representative neuron was held at various potentials from −80 to +80 mV in 20 mV increments during application of DHB. At negative holding potentials, DHB induced an outward current. The current reversed at ~0 mV and was inward at positive holding potentials. **(B)** The response of a VNO neuron to increasing concentrations of DHB. 5 ppm = 32.5 μM, 100 ppm = 650 μM. Both cells were held at −70 mV and leak subtraction was applied. The solution in the recording pipette contained (in mM): D-gluconic acid (potassium salt), 100; $MgCl_2$, 1.0; HEPES, 5.0; EGTA, 1.0; Mg/ATP, 2.0; pH = 7.25. This solution was the same for all experiments. The polished whole-cell electrode resistance ranged between 6 and 15 MΩ. The first seal resistance was measured at 6.3–22.9 GΩ. Following the second suction, the input resistance was checked regularly during the experiment to be sure it was ≤20 MΩ.

The ability of DHB to produce an outward current at negative holding potentials was related to the strength of the gigaseal. Cells with lower gigaseals were more likely to be responsive to DHB in voltage-clamp conditions than were cells with higher gigaseals, even though both populations of cells had similar voltage-gated currents. In current-clamp mode, both subsets of cells were similarly responsive.

What is the mechanism of activation?

How does DHB induce an outward current? There are two possibilities: first, the urine-derived compound might open a channel that passes outward current. Second, DHB might close a channel that passes inward current. In the first case, the input conductance of the neuron should increase, while in the second case, the input conductance should decrease. To determine the net membrane conductance, a series of small 10 mV depolarizing pulses were applied prior to, during and after the stimulus under voltage-clamp conditions. A typical response is shown in Figure 5. In this cell, DHB (6.5 μM) decreased the amplitude of the current pulses from ~50 to 30 pA (upper panel) while bath solution had no effect (lower panel). As summarized in Table 1, the current responses to the voltage pulses were significantly decreased in amplitude in the presence of DHB. These data indicate that DHB decreased net membrane conductance. Since the chemosensory stimulus produced an outward current while decreasing net membrane conductance, the underlying effect appeared to be the closing of a channel, thereby decreasing inward current.

To demonstrate that DHB reduced inward current, experiments were conducted under steady-state current conditions without leak subtraction. The results are shown in Figure 6. At potentials of −60 mV in the absence of chemosensory stimulants (baseline), there is a steady inward

current of ~–75 pA. This current was reduced to ~0 pA when DHB was added. Two other urine-derived compounds, SBT and lactol, also reduced inward current (Figure 6). All three chemosensory ligands reduced net membrane conductance as indicated by the shallower slope of the *I–V* curve compared with the slope of the baseline *I–V* plot. The decrease in net membrane conductance was

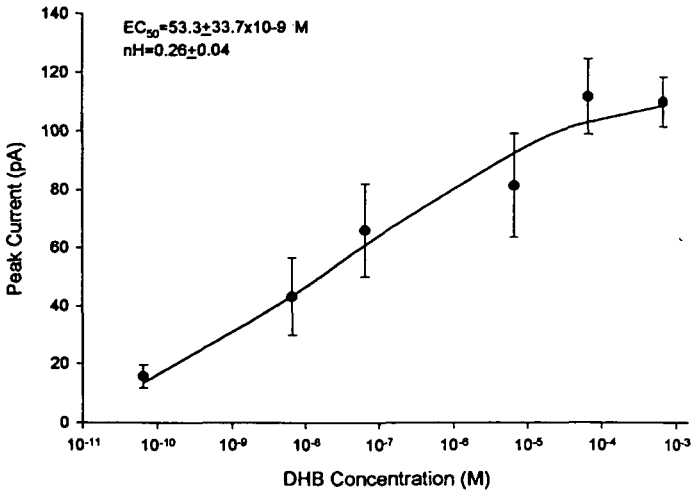

Figure 3 Dose–response curve for DHB. Each point is an average of nine cells and each cell was tested with at least four different concentrations. Data were fitted to the Hill equation and plotted with Sigma Plot. The EC_{50} and Hill coefficient are shown.

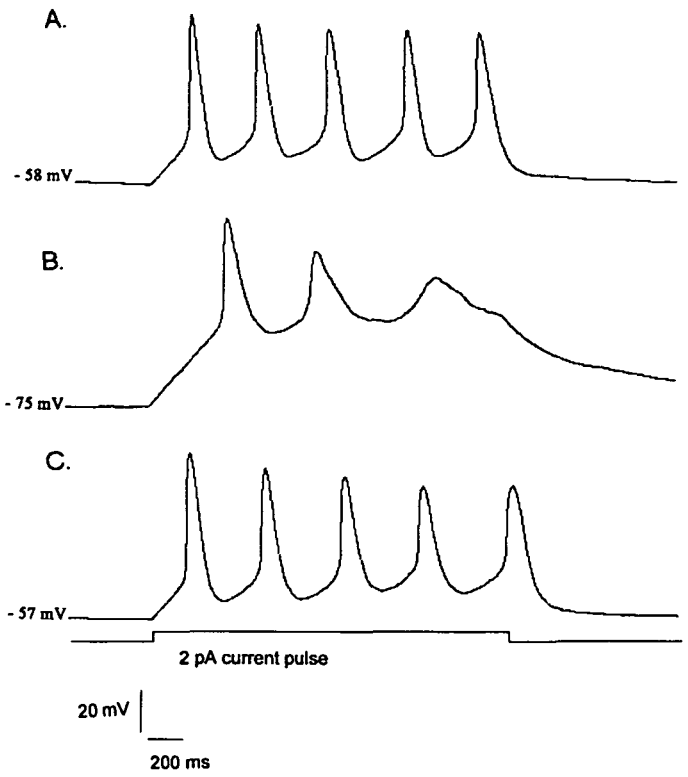

Figure 4 Response of VNO neuron to current injection in current-clamp mode. **(A)** A 2 s, 2 pA depolarizing pulse elicits repetitive firing. **(B)** Application of DHB for 2.5 min prior to and during the depolarizing current pulse reduces the number of action potential firings. **(C)** Action potential firing is recovered 15 min after the end of DHB application.

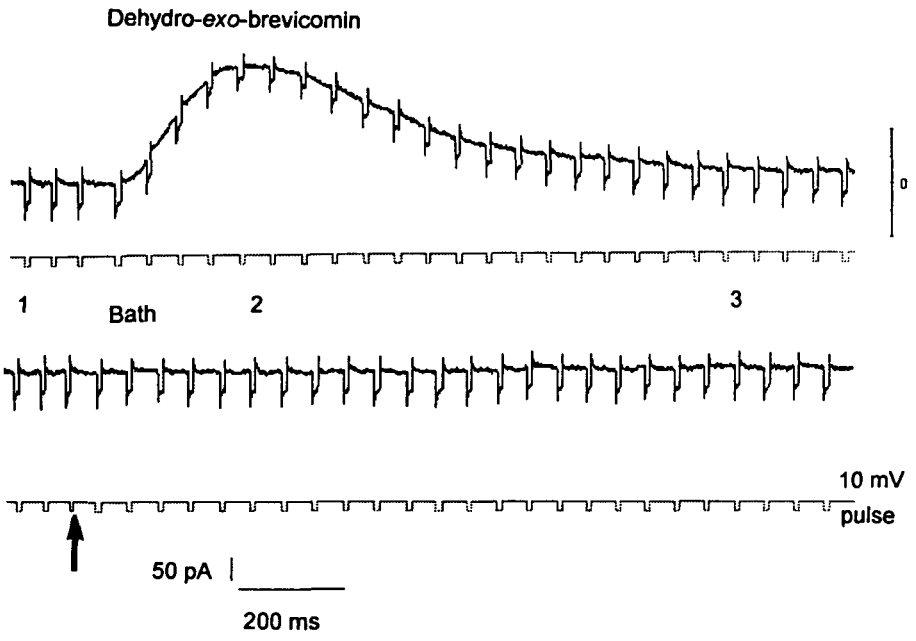

Figure 5 In voltage-clamp mode at a holding potential of –70mV, a series of 10 mV depolarizing voltage pulses were continuously applied prior to (1), during (2) and after (3) the application of either DHB or bath solution. During DHB application, the outward current was accompanied by a decrease in the amplitude of the current pulses. From Moss *et al.* (1997).

Table 1 The effect of DHB on mean membrane current, resistance and conductance

	n	Current (pA)	Resistance (GΩ)	Conductance (nS)
Bath	19	17.84 ± 2.61	0.69 ± 0.06	1.78 ± 0.26
DHB	19	13.21 ± 1.93*	1.25 ± 0.24*	1.30 ± 0.19*

Current observed in response to intracellular injection of 10 mV depolarizing pulses. Values are mean ± SEM.

*Difference between bath and DHB significant at $P \leq 0.02$ (df = 18).

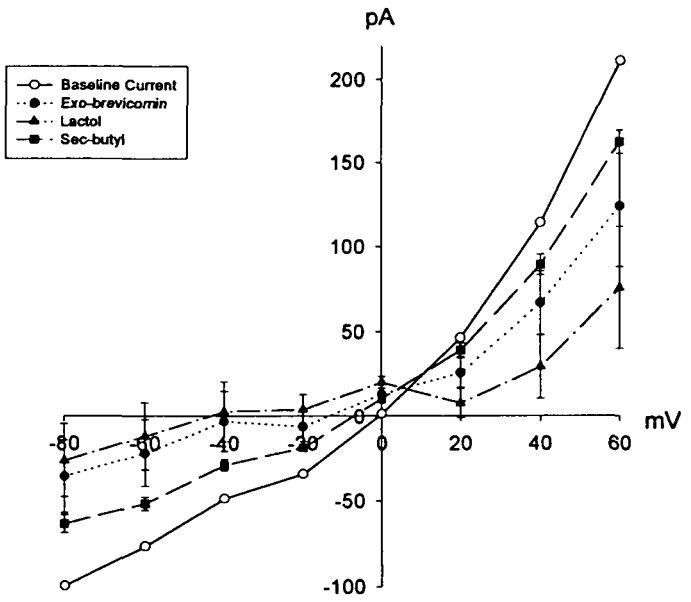

Figure 6 An I–V plot for three chemosensory ligands compared to baseline current under various holding potentials from −80 to 60 mV in 20 mV steps. No leak subtraction was applied.

voltage independent. The chemosensory agents displayed a similar reversal potential of ~0 mV.

Our data indicate that chemosensory ligands act to close a channel on VNO sensory cells, thus producing a reduction in inward current. What are the ions involved in this process? We do not know at present; however, the reversal potential of the chemosensory ligands is ~0 mV, suggesting a non-specific ion channel. Ion replacement experiments are presently in progress.

What second messenger system is involved?

Molecular biological studies have revealed that most of the molecules involved in signal transduction in the main olfactory system in mammals are not present in the VNO (Berghard *et al.*, 1996; Liman, 1996). Putative pheromone receptors in the VNO are not related to the putative odorant receptors expressed in the main olfactory epithelium (Dulac and Axel, 1995). In the main olfactory system, odor activates adenylyl cyclase type III through a $G_{\alpha OLF}$ protein (Reed, 1992; Ronnett and Snyder, 1992). Increases in cAMP open cyclic-nucleotide-gated channels composed of two subunits, oCNC1 and oCNC2 (Liman and Buck, 1994; Bradley *et al.*, 1994), leading to influx of non-specific cations, membrane depolarization and firing of action potentials (Nakamura and Gold, 1987; Firestein *et al.*, 1991). VNO neurons do not express GαOLF, adenylyl cyclase III or oCNC2 (Berghard *et al.*, 1996). Instead, two inhibitory G protein α subunits, $G_{\alpha i2}$ and $G_{\alpha 0}$ (Halpern *et al.*, 1995; Berghard and Buck, 1996), adenylyl cyclase type II (Berghard and Buck, 1996) and one subunit of the cyclic-nucleotide-gated channel (oCNC2) (Berghard *et al.*, 1996) were found in the VNO. Activation of the G proteins $G_{\alpha i2}$ or $G_{\alpha o}$ may inhibit levels of cAMP in VNO receptor cells. Based on our electrophysiological findings that application of chemosensory stimuli evoked a reduction in inward current, we hypothesized that urine-derived compounds may be acting to decrease cAMP levels in VNO neurons.

To test this hypothesis, we performed cAMP accumulation assays of the VNO after exposure to the mixture of DHB and SBT. The cAMP content was measured with the Biotrak cAMP ^{125}I assay system according to the methods indicated by the vendor. As shown in Table 2, basal levels of cAMP were not significantly different between olfactory cilia and VNO. A more precise evaluation of the VNO revealed that basal cAMP levels were not significantly different between the sensory and non-sensory portions of the organ. The mixture of DHB and SBT produced a significant decrease in cAMP levels only in tissue taken from the sensory portion of the VNO. Although the signaling mechanisms in the VNO remain unclear, the inhibitory action of DHB and SBT on cAMP levels may be exerted via a G_i-protein-coupled mechanism. Additional studies indicated that the mixture of DHB and SBT increased cAMP levels in cilia of the main olfactory epithelium (Zhou and Moss, 1997). The finding that the two urine-derived compounds had opposite effects on cAMP levels in the VNO and main olfactory epithelium lends strong support to the hypothesis that transduction mechanisms for the two types of receptor cells are different.

If VNO neurons are operating in a manner 'opposite' main olfactory receptors, then decreases in cAMP levels in VNO neurons after exposure to chemosensory ligands may indicate a closing of channels. Our electrophysiological studies also indicate that DHB closes channels to reduce inward current. No evidence was found for cyclic-nucleotide-gated channels in mouse VNO neurons (Liman and Corey, 1996), so it does not seem likely that the channels mediating signal transduction are directly gated by cAMP. A recent electrophysiological study in the rat VNO found evidence for an IP_3 pathway (Inamura *et al.*, 1997).

Table 2 Comparison of cAMP levels in olfactory cilia, whole VNO and portions of the VNO after treatement with DHB and SBT

Tissue	Treatment	cAMP (%)	*P* value
Olfactory cilia	basal	100 ± 22	
VNO	basal	78 ± 10 (3)	0.146
VNO sensory	basal	100 ± 17	
VNO non-sensory	basal	84 ± 9 (2)	0.213
VNO sensory	distilled water	100 ± 4	
VNO sensory	DHB + SBT	72 ± 1 (2)	0.001
VNO non-sensory	distilled water	100 ± 20	
VNO non-sensory	DHB + SBT	102 ± 3 (2)	0.630

Values are expressed as mean ' standard error (*n*). For each independent experiment, 9–10 mice were used. Concentration of DHB and SBT was 25 ppm.

What is the implication in chemosensory signaling?

Activation of the VN system initiates unique behavioral and physiological changes in the reproductive and social status of the animal. VN receptor neurons appear to be able to discriminate behaviorally relevant chemosensory ligands from general odorants. The chemosensory stimulants excreted in urine may evaporate and reach the nasal mucus in a volatile state or may reach the nasal mucus still bound to the MUP complex. One of the odorant-binding proteins in mouse nasal mucus, OBP-III, is identical in its first 40 amino acid sequence to MUP-5 (Pelosi, 1994; Pes and Pelosi, 1995). Two secretory proteins of the lipocalin family, vomeronasal secretory proteins I and II, as well as odorant binding protein II, are expressed in the VNO (Miyawaki *et al.*, 1994; Ohno *et al.*, 1996). These substances may act as pheromone-binding proteins and transport urine-derived compounds to the VNO.

The ease with which small amounts of current can evoke action potential generation suggests that VNO neurons may be tonically active *in vivo* in the presence of small inward currents. The reduction in inward current produced by the chemosensory ligands indicates that threshold for generation of an action potential would be harder to reach during chemosensory stimulation and thus spontaneous electrical activity would be decreased. Depending on the transmitter(s) substance(s) released by the VN sensory axon, a decrease in the electrical signals arriving in the glomeruli of the accessory olfactory bulb could either promote or inhibit electrical activity of the mitral, output cells.

Concluding remarks

Evidence accumulated from electrophysiological and molecular biology studies indicated that signal transduction mechanisms in the VNO were different from those in the main olfactory receptor neurons. Results from our electrophysiological and biochemical studies corroborate this suggestion. Chemosensory compounds derived from male urine produce an outward current at negative holding potentials accompanied by a decrease in membrane conductance. We propose that the effect of urine-derived compounds on VNO neurons is to close a channel and decrease inward current. Our biochemical studies raise the possibility that the decrease in inward current is the result of a decrease in cAMP. Obviously, additional experiments are required to test this hypothesis. Establishing the specific ion channels involved in this chemosensory response are also critical to a full understanding of transduction mechanisms in the VNO.

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