Urine-Derived Compound Evokes Membrane Responses in Mouse Vomeronasal Receptor Neurons

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Moss, Robert L., Robert E. Flynn, Xin-Ming Shen, Carol Dudley, Jiming Shi, and Milos Novotny. Urine-derived compound evokes membrane responses in mouse vomeronasal receptor neurons. J. Neurophysiol. 77: 2856–2862, 1997. Sensory neurons of the vomeronasal organ (VNO) are thought to detect species-specific chemical signals important for reproductive function. The electrical properties of VNO neurons have begun to be characterized in a variety of species; however, the response of VNO neurons to possible physiological ligands has not yet been reported. One physiological effector, dehydro-exo-brevicomin (DHB), is found in the urine of intact male mice and affects the estrous cycle of female mice. In the present study, dissociated VNO neurons were voltage- or current-clamped and their response to DHB was determined. Approximately 26% of VNO neurons responded to DHB with an outward current at negative holding potentials; the current reversed at approximately +4 mV. Application of DHB in current-clamp mode produced membrane hyperpolarization and/or a reduction in the firing of action potentials. Because membrane conductance was shown to be decreased during application of DHB, the results suggest that the outward current associated with DHB application is a reflection of a reduction in inward current caused by closing an ion channel. This study provides the first evidence that a compound found in male urine directly affects VNO neurons.

INTRODUCTION

Species-specific signals detected by sensory receptor neurons in the vomeronasal organ (VNO) are processed through the accessory olfactory system to hypothalamic centers to alter reproductive function (for review see Johns 1986; Wyscki 1979). These chemical signals, known as pheromones, are found in the urine and vaginal discharge of several rodent species. In particular, several androgen-dependent compounds isolated from male mouse urine have been demonstrated to act as attractants to female mice (Jemiolo et al. 1985), to induce estrous cyclicity in previously noncycling female mice housed in crowded conditions (Jemiolo et al. 1986), and to accelerate puberty (Mucignat-Caretta et al. 1995). More specifically, dehydro-exo-brevicomin (DHB) is found in intact male mouse urine (Wiesler et al. 1984) where it is bound to the major urinary protein (Bacchini et al. 1992). As urine is excreted and dries, DHB may be released into the air to be sniffed by conspecifics. However, because the volatility of DHB is low, this compound may contact the external nares while still in a liquid state. When mixed with another androgen-dependent compound that also binds to major urinary protein, DHB was demonstrated to induce estrous cyclicity in female mice housed in over-crowded conditions (Jemiolo et al. 1986). Although the endocrine effects of these male urine-derived compounds have been known for some time, their direct effects on sensory receptor cells of the VNO have not been examined.

Several studies examined the electrical properties of dissociated VNO neurons. In the frog, turtle, and mouse, voltage-activated inward and outward currents were recorded (Liman and Corey 1996; Taniguchi et al. 1996; Trotier et al. 1993). In all three species, action potentials were elicited by injecting small depolarizing current pulses. These studies indicated that VNO neurons possess a full array of voltage-activated currents and the electrical machinery necessary to generate action potentials; however, the responsiveness of VNO neurons to molecules likely to access the luminal fluid has yet to be determined. The present study was designed to examine the effects of the urinary compound DHB on sensory neurons of the VNO. Freshly dissociated mouse VNO neurons were studied under whole cell voltage- and current-clamp protocols to detect responses to DHB, a general depolarizing agent (KCl), or a general odorant known to alter neuronal excitability in the main olfactory epithelium (Kurahashi et al. 1994).

METHODS

Dissociation of VNO bipolar neurons

Adult female mice (20–35 g) of the ICR (Harlan) strain were rapidly decapitated, the entire bony capsule-VNO structure extracted from the nasal cavity, and the VNO dissected from the bone. VNOs were incubated in oxygenated low-Ca2+ saline solution with protease type VII (Sigma; 1.5 mg/ml) and 0.2 M urea at 22°C for 45–60 min. Urea was used to separate the supportive cells from the VNO organ was then cut open, gently raked along the concave and convex walls with a single-hair brush, and continuously oxygenated, where it is bound to the major urinary protein (Bacchini et al. 1992). As urine is excreted and dries, DHB may be released into the air to be sniffed by conspecifics. However, because the volatility of DHB is low, this compound may contact the external nares while still in a liquid state. When mixed with another androgen-dependent compound that also binds to major urinary protein, DHB was demonstrated to induce estrous cyclicity in female mice housed in over-crowded conditions (Jemiolo et al. 1986). Although the endocrine effects of these male urine-derived compounds have been known for some time, their direct effects on sensory receptor cells of the VNO have not been examined.

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In some cells the proximal portion of the axon was intact. Samples of healthy cells could be obtained for 3–4 h after dissociation.

Voltage- and current-clamp recording

Whole cell recordings were made on bipolar neurons with an Adams/List EPC-9 patch-clamp system by using a sampling frequency of 10 kHz and filtered at 2.3 kHz. The recording electrode, made from borosilicate glass, contained (in mM) 100 D-gluconic acid (potassium salt), 1 MgCl₂, 5 HEPES, 1 ethylene glycol-bis(β-aminoethly ether)-N,N,N',N'-tetraacetic acid (EGTA), and 2 MgATP, pH = 7.25. The polished whole cell patch electrode resistance ranged between 6 and 10 MΩ. The first seal resistance was measured at 6.3–22.9 GΩ. After rupture the input resistance of the VNO neuron ranged from 0.4 to 12 GΩ. The series resistance was checked regularly during the experiment to be sure it was ≤20 MΩ.

At a holding potential of ~70 mV, depolarizing and hyperpolarizing 20 mV steps were made from ~80 to 80 mV to evaluate the voltage activated inward and outward currents. Subsequently, the neuron was tested for changes in current in response to various chemical stimuli under steady-state current conditions with leak subtraction. To study the effects of DHB on net membrane conductance, voltage pulses (10 mV, 10 ms) were applied to the bipolar neuron before, during, and after the chemical application.

Delivery of test substances

The delivery of DHB (molecular weight 154.09; C₉H₁₄O₂), KCl (100 mM), a general odorant N-amyacetate (10 mM), or bath solution to the bipolar neuron was accomplished with the use of a seven-barreled puffer pipette placed near the recorded neuron. The test substances were dissolved in the bath solution and the concentration of DHB was either 6.5 μM (1 ppm) or 32.5 μM (5 ppm). The concentration of DHB in the urine of intact males is ~8 μM (1.3 ppm) (Jemiolo et al. 1986), and at this concentration, DHB mixed with the same concentration of sec-butyl-dihydrothiazole was shown to induce estrous cyclicity in female mice housed in crowded conditions (Jemiolo et al. 1986). A Medical Systems multichannel pressure unit was used to eject each individual substance onto the cell. The puffer pipette was placed 2–5 μm from the dendrite or cell body of the VN bipolar neuron and 1–8 psi was used to eject the substances for a 10-ms period. All substances were puffed onto the dendrite except for KCl, which was puffed onto the cell body or dendrite.

RESULTS

Whole cell voltage-clamp and current-clamp recordings were readily obtained from bipolar VNO neurons in the adult female mouse. To date a total of 131 neurons have been recorded. Voltage-gated inward and outward currents similar to those previously reported (Taniguchi et al. 1995, 1996; Trotier et al. 1993, 1994) were observed. Under current-clamp mode the resting membrane potential ranged from ~35 to ~75 mV. Spontaneous action potentials were observed in a number of the preparations, suggesting the possibility that bipolar VN neurons may be slightly depolarized at rest.

Typical responses to KCl, N-amyacetate, and DHB in voltage-clamp mode are seen in Fig. 1. At a holding potential of ~70 mV, application of KCl to the cell body elicited an inward current of ~375 pA. A smaller response was elicited when KCl was applied to the dendrite (Fig. 1A), indicative of a relative lack of leakage channels on the dendrite as compared with the cell body (Firestein et al. 1991). Application of N-amyacetate or the control, bath solution had no observable effect (Fig. 1B). Micropressure ejection of DHB (32.5 μM) evoked a long-lasting outward current of ~300 pA at its peak (Fig. 1C). Nearly 26% (34 of 131) of cells tested responded to DHB with an outward ionic current; no inward current was observed at negative holding potentials. The latency to response onset was 25–400 ms and was dependent on the distance between the puffer pipette and the dendrite. The response duration outlasted the stimulus application time (10 ms) by 0.8–3 s. Application of DHB to the cell body of the bipolar neurons or to nonsensory epithelial cells had no effect (data not shown).

To determine the reversal potential for the DHB effect, cells were held at ~80 to +80 mV in 20 mV increments. As seen in Fig. 2A, the current evoked by application of DHB (6.5 μM) was outward at negative holding potentials and inward at positive potentials. The mean reversal potential was 4.07 ± 1.07 (SE) mV, n = 34. The linear I-V plot (inset) indicates that the conductance was not voltage dependent. Figure 2B is an example of the effect of DHB in one cell under voltage-clamp (upper panel) and current-clamp (lower panel) modes. At a holding potential of ~70 mV, application of DHB evoked a long-lasting outward current. Under current-clamp conditions with a resting membrane potential of ~50 mV, application of DHB produced membrane hyperpolarization. With very negative resting membrane potentials (i.e., approximately ~75 mV) only a very small hyperpolarization could be observed.

The outward current observed after DHB application in voltage-clamp mode and the hyperpolarizing action of DHB on the membrane potential can be accounted for in one of two ways. Either DHB opened a channel passing outward current or it closed a channel passing inward current. In the first case the input conductance of the neuron should increase, whereas in the second case the input conductance of the neuron should decrease. The effect of DHB on membrane conductance was examined under voltage-clamp conditions by applying a series of 10 mV depolarizing voltage pulses (10 ms, 20 Hz) before, during, and after application of DHB or bath solution. A typical response is shown in Fig. 3. In this cell, DHB (6.5 μM) decreased the amplitude of the current pulses from ~50 to 30 pA (upper panel), whereas the bath solution had no effect (lower panel). Conductance in this cell was decreased from 5 to 3 nS. Similar tests comparing the current response to 10 mV depolarizing pulses during the application of bath solution and DHB were conducted in 19 neurons. On average, the conductance decreased from 1.78 ± 0.26 nS in control conditions to 1.30 ± 0.19 nS during DHB application (t = 3.89; P ≤ 0.001). These data indicate that the DHB-induced outward current was secondary to a decrease in net membrane conductance and reflective of a reduction in inward current.

Inactive agents are presently not available; however, it should be noted that DHB had no observable effect when applied to the membrane of nonsensory VNO epithelial cells (n = 10), main olfactory receptor neurons (n = 4), or CA1 pyramidal neurons of the hippocampus (n = 10).

A few cells recorded in current-clamp mode were tested for action potential generation in response to small depolariz-
**FIG. 1.** Whole cell current responses of a VNO neuron to a variety of stimuli under voltage-clamp configuration with a holding potential of \(-70 \text{ mV}\). A: response to 100 mM KCl applied to the dendrite (top trace) and to the cell body (bottom trace) of the VNO neuron. B: response to 10 mM N-amyl-acetate and bath solution applied to the dendrite of the bipolar neuron. C: response to 32.5 μM dehydro-exo-brevicomin (DHB) applied to the dendrite of bipolar neuron. DHB-induced outward current was not observed with bath solution or with N-amyl-acetate. ↑, onset of a 10-ms stimulus.

Current pulses as small as 1–2 pA induced repetitive firing of VNO neurons \((n = 3)\). In two cases, application of DHB did not alter the membrane potential but did reduce the number of action potentials firing in response to a small current pulse. In the example shown in Fig. 4A, a 2-pA current pulse induced repetitive firing of action potentials that did not accommodate during the duration of the pulse. Application of DHB for 2.5 min before and during the delivery of the current pulse decreased the number of action potential firings (Fig. 4B). In one case (not shown), a reduction in firing rate was accompanied by membrane hyperpolarization.

**DISCUSSION**

This study provides the first evidence that a compound derived from male urine can directly affect membrane cur-
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FIG. 2.  

A: whole cell current responses to DHB application at a variety of holding potentials. Cells were held at −80 to 80 mV in 20-mV steps. Reversal potential in this cell was approximately −5 mV. Inset: I-V plot of the data. B: effects of DHB in voltage-clamp and current-clamp mode. Upper panel: DHB induced an outward current under voltage-clamp conditions with a holding potential of −70 mV. Evoked outward current (150 pA) outlasted the stimulus by seconds. Lower panel: on the same cell, DHB hyperpolarized the membrane under current-clamp conditions at a resting membrane potential of −50 mV.

Dehydro-exo-brevicomin

rents in VNO neurons. DHB consistently evoked an outward current at negative holding potentials. However, DHB application also reduced net membrane conductance, indicating a reduction in current flow. Voltage clamping the membrane and applying leak subtraction balances the current at a given membrane potential to yield a net current flow of zero; however, some voltage-independent inward and outward current is still flowing. The seemingly incompatible findings of an outward current at physiological holding potentials accompanied by a reduction in net membrane conductance can be
FIG. 3. A series of 10-mV depolarizing voltage pulses was continuously applied before (1), during (2), and after (3) the application of either bath solution or DHB (6.5 μM) under voltage-clamp conditions at a holding potential of −70 mV. In the presence of DHB, the amplitude of the current pulses decreased whereas the bath solution had no effect. Current pulse amplitude before and after the DHB was ~50 pA. In the presence of DHB the current pulse amplitude decreased to 30 pA. Under control conditions the current pulse amplitude was 50 pA; this value was not effected by bath application. In the presence of DHB the resistance increased from 0.20 to 0.33 GΩ whereas the net membrane conductance decreased from 5 to 3 nS.

reconciled by hypothesizing that DHB blocks the still-flowing inward current (i.e., the ligand blocks ion channels). A reversal potential of close to 0 mV is indicative of the involvement of nonspecific cation channels and identification of the channels involved is presently underway.

Repetitive firing of action potentials in response to a small depolarizing current pulse observed in VNO neurons in the present study agrees with a previous report (Liman and Corey 1996). Although the significance of this finding is uncertain, their sensitivity to slight current changes may indicate that VNO neurons are tonically active in vivo. The present results indicate that DHB acts to hyperpolarize the membrane and/or reduce action potential firing. Such an action would lead to a reduction in the number of impulses arriving at the accessory olfactory bulb.

Urine of sexually mature, but not castrated, male mice is characterized by a high concentration of small proteins known as the major urinary proteins (MUP) (Finlayson et al. 1968; Knopf et al. 1983). The function of MUP is not known; however, ~40% of the MUPs selectively bound DHB and it was suggested that MUP may serve as a sex pheromone-binding protein (Bacchini et al. 1992). In the nasal passages and VNO of the mouse and rat, several proteins with odorant binding characteristics have been identified (Miyawaki et al. 1994; Ohno et al. 1996; Pelosi 1994; Pes and Pelosi 1995). Many of these odorant binding proteins possess high sequence homology to MUP (Pes and Pelosi 1995). A pheromone released from MUP could bind to a structurally similar protein in the nasal cavity or VNO for transport to receptor sites. Although the ligands for binding proteins localized in the VNO have not yet been identified, the present data indicate that DHB may be such a ligand.

The action of DHB to decrease net conductance is quite different from the increased conductance produced by general odorants in the main olfactory bulb (Dionne and Dubin 1994). Differences in electrophysiological properties and cyclic nucleotide-gated channels between olfactory receptor neurons and VNO neurons were reported. The voltage-activated K⁺ currents activated more slowly in VNO neurons than in neurons from the main olfactory epithelium (Liman
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FIG. 4. Response of a VNO neuron to current injection in current-clamp mode. A: 2-s depolarizing pulse elicits repetitive action potential firing. B: constant puffing of DHB (32.5 μM) for 2.5 min before and during the depolarizing current pulse reduces the number of action potential firings. C: action potential firing is recovered 15 min after DHB application was ended.

A

-62 mV

B

-61 mV

C

-62 mV

2 pA current pulse

20 mV

200 ms

and Corey 1996). In current-clamp mode, small depolarizing pulses elicited repetitive firing of VNO neurons with no sign of adaptation (Liman and Corey 1996; Taniguchi et al. 1996; Trotier et al. 1993), whereas most olfactory receptor neurons fire only one action potential in response to depolarizing current pulses (Dubin and Dionne 1994) or, if repetitive firing is observed, stronger depolarizing current is necessary (Leinders-Zufall et al. 1995) and action potentials further along in the series show decreasing amplitudes (Hedlund et al. 1987; Schmiedal-Jakob et al. 1989). An electrophysiological study in the mouse found no evidence for cyclic nucleotide-gated channels in VNO neurons (Liman and Corey 1996). Analysis with Northern blot and in situ hybridization techniques revealed that molecules involved in signal transduction in the main olfactory system were not expressed in VNO neurons (Berghard and Buck 1996). Furthermore, putative receptor genes identified in the VNO were not related to the receptors expressed in the main olfactory epithelium (Dulac and Axel 1995). Thus signal detection, integration, and transduction in VNO neurons appears to be very different from mechanisms used in main olfactory receptor cells.

The action of DHB at the VNO neuron is apparently an example of the unusual situation of a receptor neuron firing less when a stimulus is applied. Although the effects of urinary compounds on VNO neurons in vivo remain to be elucidated, the effects observed on dissociated VNO neurons are reminiscent of transduction mechanisms involved in the visual (Baylor 1992; Baylor and Nunn 1986) and lobster olfactory receptor (Fadool and Ache 1992; Hatt and Ache 1994; Michel and Ache 1992, 1994) systems. The physiological significance of a reduction in transmitter release at VNO terminal endings in the accessory olfactory bulb after pheromone exposure awaits identification of the transmitters involved.

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