Urine Stimulation Activates BK Channels in Mouse Vomeronasal Neurons

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Zhang P, Yang C, Delay RJ. Urine stimulation activates BK channels in mouse vomeronasal neurons. J Neurophysiol 100: 1824–1834, 2008. First published August 13, 2008; doi:10.1152/jn.90555.2008. Most odor responses in mouse vomeronasal neurons are mediated by the phospholipase C (PLC) pathway, activation of which elevates diacylglycerol (DAG). Lucas et al. showed that DAG activates transient receptor potential channels, subfamily C, member 2 (TRPC2), resulting in a depolarizing Ca2+ influx. DAG can be subsequently converted to arachidonic acid (AA) by a DAG lipase, the role of which remains largely unknown. In this study, we found that urine stimulation of vomeronasal neurons activated large-conductance Ca2+-activated K+ (BK) channels via AA production. Using isolated neurons, we demonstrated that repetitive applications of AA potentiated a K+ current that required a Ca2+ influx and was sensitive to specific BK blockers. Using immunocytochemistry, we found that BK channels are present in vomeronasal neurons with labeling on the soma and heavy labeling on the dendrite with a BK channel antibody. We examined the role of these BK channels in regulating neuronal firing when the neuron was activated by membrane depolarization or urine. Contrary to a recent report, our data suggest that BK channels contribute to adaptation of urine/odor responses because the inhibition of BK channels during odor stimulation promoted repetitive firing. These data strongly support the hypothesis that AA mediates an inhibitory pathway through BK channels, a possible mechanism for odor adaptation in vomeronasal neurons.

INTRODUCTION

In mice and many other vertebrates, odors are detected in at least three locations: the main olfactory epithelium (MOE), septal organ (SO), and vomeronasal organs (VNOs) (reviewed in Breer et al. 2006). Neurons from the MOE and SO seem to function similarly, with odor responses mainly mediated by increases in intracellular cAMP (Ma et al. 2003). Neurons from the VNOs detect odors (reviewed in Baxi et al. 2006), chiefly via the phospholipase C (PLC) pathway (Inamura et al. 1997; Krieger et al. 1999; Wekesa and Anholt 1997). Odor activation of PLC pathway results in the elevation of two second messengers, diacylglycerol (DAG) and IP3, with DAG activating the Ca2+-permeable channel, TRPC2 channel (Liman et al. 1999; Lucas et al. 2003). DAG can be converted to another second messenger, AA, by DAG lipase. It remains controversial whether AA plays a role in odor transduction of vomeronasal neurons. A Ca2+-imaging study by Spehr et al. (2002) found that blockade of AA synthesis by a DAG lipase inhibitor decreased urine-induced Ca2+ transients, but this observation contradicts data from a whole cell patch-clamp study (Lucas et al. 2003), which argued that the DAG lipase inhibitor had no effect on urine-activated inward currents. Nonetheless both Ca2+ imaging and electrophysiological studies suggest that increases in AA elicit a Ca2+ influx in vomeronasal neurons that does not involve TRPC2 channels (Lucas et al. 2003; Spehr et al. 2002). These findings raise critical questions about the role of AA in vomeronasal neurons. For example, does AA contribute to odor transduction of vomeronasal neurons and, if so, what is its role?

In numerous tissues AA activates BK channels and serves a variety of functions. For example, in rabbit coronary smooth muscle cells, AA activates BK channels and contributes to the ischemic coronary vasodilatation (Ahn et al. 1994). In bovine adrenal chromaffin cells, AA activates BK channels and inhibits secretion (Twitchell et al. 1997). Furthermore, BK channels are expressed in olfactory sensory neurons (OSNs) of rat (Rattus norvegicus) and toad (Caudiverbera caudiverbera) and are implicated in inhibitory odor responses (Castillo et al. 2005; Delgado and Bacigalupo 2004; Delgado et al. 2003; Kawai 2002; Morales et al. 1995, 1997). It was not until recently that Ukhanov et al. (2007) detected the presence of BK conductance in mouse vomeronasal neurons and suggested that it promotes persistent neuronal firing. However, the region of expression, modulation and function of BK channels in vomeronasal neurons remain largely unknown.

In this study, we found heavy labeling with an antibody specific to BK channels at the dendrite, dendrite knob and apical soma of mouse vomeronasal neurons. We also found that BK current was subject to modulation by AA and that inhibition of BK current increased the firing frequency of action potentials in vomeronasal neurons. Our data support the hypothesis that BK channels are activated during odor stimulation and that ion flux through these channels limit the number of action potentials generated in response to odors.

METHODS

Experimental animals

We used 4 to 12-wk-old, male and female mice of two strains, C57BL/6 (Charles River, Wilmington, MA) and one strain expressing OMP-GFP chimeras (OMP, olfactory marker protein, is exclusively expressed in mature vomeronasal and olfactory neurons). OMP-GFP mice were a kind gift from Peter Mombaerts, Rockefeller University. Mice were maintained in the Animal Care Facility at the University of Vermont in accordance with IACUC guidelines.

Urine collection

The urine was collected by gently pressing the abdomens of mice. A urine mixture was made by combining the urine from ≥30 male and female mice. The urine mixture was filtered through a 0.2 μm syringe filter (Fisher, Pittsburgh, PA) and stored at −70°C for ≤3 mo. Prior
to use, the urine mixture was diluted in Ringer (1:200) and the pH adjusted to 7.4.

Preparation of isolated vomeronasal neurons

The vomeronasal neurons of either C57BL/6 or OMP-GFP mice were acutely dissociated by a modified version of a previously reported method (Spehr et al. 2002). Briefly, mice were killed with CO₂ followed by cervical dislocation. The VNOs were dissected out, cut into small pieces, and incubated with 50 μg/ml papain (Calbiochem, La Jolla, CA) in a divalent cation-free Ringer solution for 15 min at room temperature. Cells were gently pipetted, filtered through a 250-μm nylon mesh (Small Parts, Miami Lakes, FL), and transferred into a fresh Ringer solution containing 10 μg/ml leupeptin (USB, Cleveland, OH). Isolated cells were kept at room temperature and usually used within 4 h of preparation.

Immunocytochemistry

OMP-GFP mice were used for immunocytochemistry with a BK antibody (rabbit anti-mouse) that recognizes the intracellular C-terminus (amino acid residues 1098-1196; APC-021, Alomone Labs, Jerusalem, Israel). Immunostaining of both VNO sections and isolated cells was performed. To obtain the sections, VNOs were dissected out from their bony capsule and fixed in 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) in 0.1 M phosphate buffer for 90 min, washed with 0.1 M phosphate buffer, and cryoprotected by running through a graded sucrose series (0.5, 1.0, 1.5, and 2.0 M). The tissue block was mounted on a HM505E cryostat (Microm, Walldorf, Germany) and sectioned at 10–20 μm. For immunocytochemistry on isolated cells, freshly isolated cells were plated onto a Concanavalin A-coated microscope cover glass and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min. After fixation, sections or isolated cells were blocked with 4% normal goat serum and 0.2% Triton X-100 in PBS. This was followed by incubation overnight in a 1:200 dilution of BK antibody (or in a BK antibody preabsorbed with control BK antigen for 1 h to confirm antibody specificity), washed in PBS, and treated with 1:200 dilution of Rhodamine Red-X goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 h. The samples were imaged with a laser scanning confocal microscope (Zeiss LSM 510 Meta).

Electrophysiology

Perforated patch clamp was performed using gramicidin, which is permeable to Na⁺ and K⁺ but not to Cl⁻ (Myers and Haydon 1972). Gramicidin was dissolved in DMSO and mixed into the intracellular solution (0.22 mg/ml) by vortex for 10 min and brief sonication. Gramicidin was dissolved in DMSO and mixed into the intracellular solution minus gramicidin and then back-filled with gramicidin-containing intracellular solution. After gigaseals were formed on isolated vomeronasal neurons, diffusion of gramicidin to the membrane was monitored by the appearance of voltage-activated currents as access to the interior of cells was established. After establishing voltage control, both voltage- and current-clamp recordings were performed. For the voltage-clamp experiments, the cells were held at −60 mV and pulsed in 11 voltage steps from −100 to +100 mV in 20-mV increments for 5 s per step returning to −60 mV for 150 ms between each voltage step. In the middle of each voltage step, 50 μM AA was applied for 2 s using a SF-77B fast perfusion stepper system (Warner Instruments, Hamden, CT). In many of the current traces, an artifact, caused by the stepper system can be observed at the start and end of each stimulus. At each voltage step, the AA-activated current was determined by subtracting the plateau current before AA stimulation from the peak response to AA. When repetitive stimulation was employed, the 11 voltage step sequence was repeated 5–10 times with 10-s interval separating each sequence. Immediately following recordings of AA-activated currents in Ringer, the same cell was exposed to bath-applied drugs [for example: TEA, iberiotoxin (IbTX), Cd²⁺, thapsigargin and 2-aminoethoxy diphenyl borate (2-APB)] and stimulated with AA plus drug using the same protocol. The AA-activated currents (in Ringer/drugs) were normalized to the current at +100 mV in Ringer (100%) for the I-V plots. For current clamp, action potentials were stimulated by current injection or by urine (1:200) applied through the perfusion system. IbTX (100 nM) was applied through bath perfusion to assess changes in the stimulated neuronal firing. Electrophysiological data were acquired using MultiClamp 700A, Digidata 1322A, and pCLAMP 8.2 software (Axon Instruments, Union City, CA).

Calcium imaging

Calcium imaging was performed with a Zeiss Axioskop 2FS or a Nikon Eclipse TE200 microscope. Isolated C57BL/6 mouse vomeronasal neurons were placed on Concanavalin A-coated microscope cover slips fitted in a recording chamber. The cells were allowed to settle and attach to glass for 10 min. Cells were loaded with Fura 2-AM (Molecular Probes, Eugene, OR), a Ca²⁺-sensitive dye, for 30 min at room temperature (see Solutions). The dish was washed with a constant flow of Ringer solution for 10 min before testing to completely wash off the Fura 2-AM. The vomeronasal neurons were identified using a 40×-phase objective and images taken with an Orca100 digital camera. Cells were illuminated every 8 s for 50–200 ms at 340 and 380 nm. The average intensity in the selected regions of interest was captured with either Open Lab 3.5.1 software (Improvision, Lexington, MA) or SimplePCI 5.0 software (Compix, Cranston, MA). The dish was washed with a constant flow of Ringer solution for 10 min before testing to completely wash off the Fura 2-AM. The vomeronasal neurons were identified using a 40×-phase objective and images taken with an Orca100 digital camera. Cells were illuminated every 8 s for 50–200 ms at 340 and 380 nm. The average intensity in the selected regions of interest was captured with either Open Lab 3.5.1 software (Improvision, Lexington, MA) or SimplePCI 5.0 software (Compix, Cranston, MA).
berry Township, PA). The Ca\(^{2+}\)-dependent fluorescence signal was expressed as a F340/F380 ratio. Cells were stimulated with 50 \(\mu\)M AA through the perfusion system, either by a single 60-s application or using the same repetitive application strategy as stated in the preceding text for perforated patch clamp, i.e., 2-s AA stimulations every 5 s for 11 times in each round of AA stimulations.

**Solutions**

All solutions were adjusted to pH 7.4. Ringer solution consisted of (in mM) 138 NaCl, 10 HEPES, 10 glucose, 5 KCl, 2 MgCl\(_2\), and 2 CaCl\(_2\). For cell dissociation, a divalent cation-free Ringer consisted of (in mM) 140 NaCl, 10 HEPES, 10 glucose, and 5 KCl was used. Ca\(^{2+}/\text{Na}\(^{+}\)-free Ringer consisted of: 145 choline.Cl, 10 HEPES, 10 glucose, 5 KCl, 2 MgCl\(_2\), and 5 EGTA. High-K\(^{+}\) Ringer solution consisted of (in mM): 80 NaCl, 65 KCl, 10 HEPES, 10 glucose, and 2 CaCl\(_2\). Mouse intracellular solution consisted of (in mM) 110 K-gluconate, 30 KCl, 10 NaCl, 10 HEPES, 0.023 CaCl\(_2\), 1 MgCl\(_2\), and 1 EGTA. The Fura 2-AM solution was made to a final concentration of 4 \(\mu\)M with 0.0083% pluronic F127 added. Phosphate buffer saline (PBS) consisted of (in mM) 2 NaH\(_2\)PO\(_4\), 8.4 Na\(_2\)HPO\(_4\), and 150 NaCl. 0.1 M phosphate buffer was diluted from a 0.2 M phosphate buffer stock solution; 0.2 M phosphate buffer was made by combining 0.2 M NaH\(_2\)PO\(_4\) and 0.2 M Na\(_2\)HPO\(_4\) in a ratio to reach pH 7.4. All chemicals and drugs were bought from Sigma-Aldrich (St. Louis, MO) if not specified otherwise.

**Statistical analyses**

Quantitative data are expressed as means ± SE. Statistical analyses were performed using SigmaStat 3.5 (Systat Software, San Jose, CA). Statistical significance was assessed using a paired or unpaired Student’s t-test or a two-way repeated-measures ANOVA analysis followed by Tukey pair-wise multiple-comparison post hoc test. *P* < 0.05 was considered as significant. F values of ANOVA analyses were expressed as \(F(x, y, n)\) of interaction between bath solutions (Ringer vs. drug, \(df = 1\)) and voltages \((−100 \sim +100 \text{ mV})\), 11 steps, \(df = 10\), where \(x\) and \(y\) are the degree of freedom (\(df\) of interactions (\(x = 10^*1\)) and the \(df\) of error terms [\(y = 10^*(n − 1)\); \(n\): number of cells], respectively.

**RESULTS**

AA is generated by urine stimulation and plays a role in odor responses. Spehr et al. (2002) suggested that AA is produced during odor responses in vomeronasal neurons and can increase intracellular Ca\(^{2+}\) when bath applied. To confirm the involvement of AA in odor responses, we stimulated vomeronasal neurons for 0.5 s with diluted urine (1:200). Urine activated an inward current of 34.7 ± 9.6 pA at −80 mV (means ± SE of 7 cells from 6 mice; Fig. 1A, control) presumably via odor-activated second messengers (DAG and/or AA). Blockade of AA synthesis by a DAG lipase inhibitor, 50 \(\mu\)M RHC80267, resulted in ~50% reduction of urine-induced inward currents [15.4 ± 6.7 pA, 7 cells from 6 mice, \(t(6) = 5.2, P = 0.002\), paired Student’s t-test], which suggests that AA is produced and functions in odor responses of vomeronasal neurons (Fig. 1). The remaining part of urine-induced current in RHC80267 should be via DAG-activated TRPC2 channels (Lucas et al. 2003).

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**FIG. 2.** AA elicited an outward current and repetitive application potentiated it. A, top inset: protocol used. Briefly, voltage-clamped cells were pulsed with 11 voltage steps (−100 to +100 mV in 20-mV increments, 5 s/step) and exposed to AA for 2 s at each voltage step. This 11-voltage-step recording series was repeated until the response no longer potentiated. For clarity, only the responses at +100 mV are shown (same in Figs. 3–6). Repetitive application of 50 \(\mu\)M AA increased an outward current. The initial response was small (the 4th round of recordings) and the repetitive application potentiated the response (the 10th round of recordings). When Ringer was applied instead of AA (control), the outward current kept relatively constant. B: the corresponding I-V plots of the 4th and 10th round of recordings shown in A. C: the AA-activated outward current at each voltage was calculated by subtracting the basal current level before AA application from the peak current elicited by AA. Repetitive application of AA induced an outward current starting from about −40 mV that increased at more depolarizing testing potentials. C: the time course of AA-activated outward current at +100 mV. Data are calculated from 11 cells of 11 mice and are expressed as means ± SE. The response was small initially, potentiated and reached a plateau with repetitive application of AA.
Repetitive stimulation with AA induces an outward K⁺ current

To elucidate the role of AA, especially its effect on the membrane conductance of vomeronasal neurons, we performed voltage clamp using a 11-voltage-step protocol that repetitively stimulated cells with AA and recorded AA-activated currents from −100 to +100 mV (Fig. 2A, inset, see METHODS). For clarity, only the responses to AA at +100-mV

FIG. 3. The AA-activated current was a K⁺ current. A: the AA-activated current was largely suppressed by 5 and 20 mM TEA at +100 mV. B: the I-V plots of AA-induced responses in Ringer and 5 and 20 mM TEA. Each cell was bathed in both Ringer and ≥1 concentration of the TEA (13 cells from 13 mice in total, 8 were tested in 5 mM TEA and 9 were tested in 20 mM TEA). For each cell, the AA-activated currents were normalized to the response at +100 mV in Ringer (100%). The I-V relationships under each condition were averaged and plotted as means ± SE. Two-way ANOVA with pair-wise multiple comparison test revealed that both 5 and 20 mM TEA significantly reduced AA-activated responses from −20 to +100 mV (*P < 0.05), suggesting that AA activated a K⁺ current.

Repetitive stimulation with AA induced an outward K⁺ current

FIG. 4. The AA-activated current was Ca²⁺-dependent. A: a nonspecific Ca²⁺ channel blocker, 1 mM Cd²⁺ blocked the AA-activated current at +100 mV. B: the I-V plots of AA-induced responses in normal Ringer and Cd²⁺ Ringer (5 cells from 5 mice). Cd²⁺ significantly reduced AA-activated responses from −40 to +100 mV (*P < 0.05, 2-way ANOVA). C: removing extracellular Ca²⁺ (0 Ca²⁺) greatly reduced the AA-activated current at +100 mV (Na⁺ was substituted with choline⁺ to stabilize the recording). D: the I-V plots of AA-induced responses in normal Ringer and 0 Ca²⁺ Ringer (7 cells from 4 mice). The AA-activated current was significantly decreased by removing extracellular Ca²⁺ from −20 to +100 mV (*P < 0.05, 2-way ANOVA). E: a sarcoendoplasmic reticulum calcium transport ATPase (SERCA) blocker, 2 μM thapsigargin did not block the AA-activated current at +100 mV. F: the I-V plots of AA-induced responses with and without thapsigargin (4 cells from 3 mice). Thapsigargin did not block but rather increased the AA-activated current at above +60 mV (*P < 0.05, 2-way ANOVA).
pulse are shown in Fig. 2A and the following figures. The magnitudes of AA responses at other voltages are shown in the corresponding I-V curves. We found that AA activated an outward current in over 90% of cells tested (136 of 145 cells, Fig. 2, A and B). The AA-induced currents were an outwardly rectifying current activated at above approximately −40 mV, suggesting a voltage dependency of the AA-activated channels (Fig. 2B). Higher level of noise was observed in the presence of AA probably due to open channel noise. Interestingly, in the first few stimulations with our recording procedure, AA only slightly increased the outward currents; but after several repetitions of the protocol, the responses were usually potentiated (Fig. 2, A and B). A plateau effect was reached after ~10 successive repeats of the 11-voltage-step protocol (Fig. 2C, 11 cells from 11 mice).

The AA-activated currents appeared to be a K⁺ current because it was blocked by a nonspecific K⁺ channel blocker, tetraethylammonium (TEA) at 5 mM [8 cells from 8 mice; interaction of bath solutions by voltage steps: F(10,70) = 71.4, P < 0.001, 2-way ANOVA] and 20 mM [9 cells from 9 mice; interaction of bath solutions by voltage steps: F(10,80) = 251.5, P < 0.001, 2-way ANOVA; Fig. 3]. A pair-wise multiple comparison test revealed that both 5 and 20 mM TEA significantly reduced AA-activated currents at −20 mV and higher voltages (P < 0.05; Fig. 3B).

**AA-activated currents are Ca²⁺ dependent**

The AA-activated outward currents displayed Ca²⁺ dependency as shown when we used two ways to affect Ca²⁺ influx. First, we added a nonspecific Ca²⁺ channel blocker, 1 mM Cd²⁺ to the bath (Fig. 4, A and B). Cd²⁺ blocks L-type Ca²⁺ channels and several other Ca²⁺-permeable channels, including Ca²⁺-activated nonselective cation ion channels (Chang et al. 1995; Haj-Dahmane and Andrade 1997; Nakajima et al. 1996) that are present in vomeronasal neurons (Liman 2003). We found that Cd²⁺ effectively abolished the AA-activated K⁺ currents [5 cells from 5 mice; interaction of bath solutions by voltage steps: F(10,40) = 135.8, P < 0.001, 2-way ANOVA; Fig. 4, A and B], and the effect of Cd²⁺ was reversible (data not shown). Second, we removed the extracellular Ca²⁺ using a Ca²⁺ chelator, EGTA (0 Ca²⁺). The recordings in 0 Ca²⁺ solution were unstable and extremely noisy (data not shown), so we used a bath solution containing neither Ca²⁺ nor Na⁺ (Na⁺ was substituted with choline, see Solutions) to reduce the noise. In this Ca²⁺/Na⁺-free solution, AA-activated currents are Ca²⁺ dependent as shown when we used two ways to affect Ca²⁺ influx.

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**FIG. 5.** Repetitive AA application induced a prolonged elevation of the intracellular Ca²⁺. A, left: a DIC image of an isolated vomeronasal neuron, which has a dendrite extending from soma. The microvilli are located in the "fuzzy" region at the end of the dendritic knob. **Middle and right:** pseudo-colored images of the cell loaded with Fura-2. **Top inset:** the color scale in correspondence to Ca²⁺ level (F340/F380). Application of a high-K⁺ Ringer depolarized the membrane potential, resulting in an increase in the intracellular Ca²⁺ (right). B, in a representative cell that is shown in the right, Ca²⁺ imaging was performed to measure the intracellular Ca²⁺ on the dendritic knob region (red circle). AA (50 μM) was repetitively applied with the same protocol illustrated in the Fig. 2. As a result, a prolonged Ca²⁺ elevation was elicited by the repetitive application of AA (15 cells from 8 mice). C, in another representative cell, repetitive application of Ringer using the same protocol elicited no change in [Ca²⁺], (8 cells from 2 mice).
the AA-activated current was greatly reduced [7 cells from 4 mice; interaction of bath solutions by voltage steps: $F(10,60) = 47.8$, $P < 0.001$, 2-way ANOVA; Fig. 4, C and D].

The data suggest that AA activates a $\text{Ca}^{2+}$ influx across plasma membrane, which in turn activates a $\text{Ca}^{2+}$-dependent channel. By contrast, $\text{Ca}^{2+}$ stores in the endoplasmic reticulum seemed not to be involved in AA-activated responses because thapsigargin (2 $\mu$M), a sarcoplasmic-endoplasmic reticulum calcium ATPase inhibitor that depletes internal $\text{Ca}^{2+}$ stores, did not block AA-activated currents (4 cells from 3 mice; Fig. 4, E and F). This is consistent with a $\text{Ca}^{2+}$ imaging study that showed no effect of thapsigargin on the AA-activated $\text{Ca}^{2+}$ transients (Spehr et al. 2002). Interestingly, thapsigargin actually increased the AA-activated currents by $\sim 20\%$ interaction of bath solutions by voltage steps: $F(10,30) = 5.0$, $P < 0.001$, 2-way ANOVA]. A pair-wise multiple comparison test indicated that thapsigargin significantly increased the AA-activated currents between $+60$ and $+100$ mV ($P < 0.05$; Fig. 4F), probably because thapsigargin increased the cytoplasmic $\text{Ca}^{2+}$.

To confirm that AA activates a $\text{Ca}^{2+}$ influx, we performed a $\text{Ca}^{2+}$ imaging study (Fig. 5). We found that the application of 50 $\mu$M AA for 60 s increased intracellular $\text{Ca}^{2+}$ in about a third of the cells tested (6/15 cells from 10 mice, data not shown). To match the stimulation protocol in our electrophysiological recording (Fig. 2), we used the same repetitive application strategy to stimulate vomeronasal neurons in $\text{Ca}^{2+}$ imaging experiments. Similar to a single 60-s application of AA, multiple rounds of repetitive AA application induced a prolonged $\text{Ca}^{2+}$ elevation in both the dendrite knobs and soma of most of the cells tested (Fig. 5B, 15/18 cells from 8 mice). As a control, repetitive applications of Ringer did not affect intracellular $\text{Ca}^{2+}$ in any of the cells tested (Fig. 5C, 8 cells from 2 mice). In addition, AA was not able to elicit $\text{Ca}^{2+}$ transients in olfactory neurons (data not shown), suggesting that such AA-activated $\text{Ca}^{2+}$ signaling was not generic in vomeronasal neurons. Consistent with the electrophysiological data, $\text{Ca}^{2+}$ images of AA responses were not in the millisecond but in the second range, suggesting a possible connection between the AA-induced $[\text{Ca}^{2+}]_i$ increase and the activation of the outward currents by AA (Figs. 2C and 5B, see DISCUSSION).

FIG. 6. The AA-activated $\text{K}^+$ current was blocked by BK channel blockers, but not by a SK channel blocker, indicating that it was a BK current. A and C: both BK channel blockers, 100 nM iberiotoxin (IBTX, A) and 100 nM iberiotoxin (CHTX, C), inhibited the AA-activated outward current at $+100$ mV. B and D: the I-V plots of AA-activated currents in the presence of IBTX (6 cells from 6 mice) and CHTX (5 cells from 3 mice), respectively. The AA-activated outward current was almost completely blocked by both IBTX and CHTX, suggesting that the repetitive application of AA activated a BK current (*$P < 0.05$, 2-way ANOVA). E: the SK channel blocker (apamin, 1 $\mu$M) did not inhibit the AA-activated outward current at $+100$ mV. F: the I-V plots of AA-induced responses in Ringer and apamin (5 cells from 5 mice). At all testing potentials, apamin did not affect the AA-induced current, suggesting that the current was not through SK channels ($P > 0.05$ at all voltages, 2-way ANOVA).
AA-activated outward currents are through BK channels

To further characterize this Ca\(^{2+}\)-dependent K\(^+\) current we found that the AA-activated currents were blocked by two specific BK channels blockers, 100 nM IbTX [6 cells from 6 mice interaction of bath solutions by voltage steps: 10(10, 50) = 69.1, \(P < 0.001\), 2-way ANOVA] and 100 nM charybdotoxin [ChTX, Calbiochem, La Jolla, CA] cells from 5 mice interaction of bath solutions by voltage steps: 10(10,40) = 61.3, \(P < 0.001\), 2-way ANOVA; Fig. 6, A–D]. This suggests that AA activated BK channels selectively. In contrast, a SK (small-conductance Ca\(^{2+}\)-activated K\(^+\)) channel blocker, 1 mM apamin, had no effect on the AA-activated currents [5 cells from 5 mice interaction of bath solutions by voltage steps: 10(10,40) = 1.33, \(P = 0.25\), 2-way ANOVA; Fig. 6, E and F]. Multiple comparison test revealed no effect of apamin on the AA-activated currents at all voltages (\(P > 0.05\); Fig. 6F).

BK channels are expressed in vomeronasal neurons close to the site of odor transduction

To study the cellular localization of BK channels on vomeronasal neurons, we performed BK antibody staining on both coronal sections of the VNOs (Fig. 7) and dissociated vomeronasal neurons (Fig. 8) using tissue from OMP-GFP mice. As expected, the OMP-GFP fluorescence, a positive marker for sensory epithelium in VNO sections (SE, concaved side) where vomeronasal neurons are located (Fig. 7, A and D). BK labeling co-localized with OMP-GFP fluorescence and showed a higher level of expression near the apical surface where the dendrite knobs and microvilli are located. In contrast, no BK labeling was detectable in the nonsensory epithelium (NSE, convex side), except for some light autofluorescence (Fig. 7B). The overlapped BK and OMP-GFP labeling strongly supports the presence of BK channels in vomeronasal tissue (Fig. 7C). As a control, no BK labeling was detected in VNOs when omitting BK antibody (Fig. 7, D–F) or using BK antibody that was preabsorbed with a control BK antigen for 1 h (data not shown). However, there was also BK labeling in what appeared to be supporting cells that lie between the dendrites of vomeronasal neurons near the apical surface (Fig. 7C). This made it difficult to determine if the BK label was really in vomeronasal neurons or just in the supporting cells. To resolve this problem, we stained isolated cells (Fig. 8). Vomeronasal neurons were identified by their morphology with DIC optics (Fig. 8, A and E) and expression of OMP (Fig. 8, B and F). We repeated immunostaining four times (\(n = 15\) cells, 4 different animals), all of which showed BK staining. BK channels were expressed in the soma, dendrites and probably also in the microvilli (i.e., “fuzzy” region), with a comparably high expression in the apical soma and dendrite knobs (Fig. 8C). In contrast, no labeling could be detected in the control with omission of BK antibody (Fig. 8G, we repeated these experiments three times (\(n = 10\) cells, 3 different animals) and found no staining on any of the control cells.

Odors activate BK channels and inhibition of BK channels promotes repetitive firing

Ukhanov et al. (2007) speculated that BK channels promote persistent firing of vomeronasal neurons, based on the observation that a high concentration of IbTX (1 \(\mu\)M) inhibited repetitive firing of vomeronasal neurons. Because the IC\(_{50}\) (half-maximal concentration) of IbTX is only \(~10\) nM (Liu et al. 2002; Ransom and Sontheimer 2001), 1 \(\mu\)M is quite high and might have nonspecific effects. To better assess the role of BK channels in stimulated vomeronasal neurons, we performed current clamp experiments using a moderate concentration of IbTX (100 nM) that was sufficient to block BK channels (Fig. 6, A and B) and has been consistently used by other researchers (Fernandez-Fernandez et al. 2002; Holland et al. 1996; Kraft et al. 1999).
2000). Under our recording conditions, we observed that stimulation with either current injection or dilute urine in the presence of 100 nM IbTX actually increased repetitive action potentials (APs) of vomeronasal neurons (Figs. 9 and 10).

We first elicited APs of vomeronasal neurons by injecting various amounts of current for 1 s (Fig. 9). Consistent with previous reports (Liman and Corey 1996; Shimazaki et al. 2006; Ukhanov et al. 2007), injection of only a few picoampere...
current elicited repetitive firing and larger current injections increased the firing frequency in vomeronasal neurons (Fig. 9A). The role of BK channels was assessed by applying 100 nM IbTX to the bath. In the majority of cells tested, IbTX increased the frequency of APs (Fig. 9B, 5/6 cells from 6 mice, only 1 cell showed no effect with IbTX). By comparing the shape of APs with and without IbTX, it appears that IbTX shortened the afterpotential hyperpolarization, so that the membrane potential reached threshold quicker and the firing frequency increased (Fig. 9C). To quantify the effects of IbTX, we calculated three parameters of APs elicited by a 1-s injection of 12-pA current from the five cells tested under both control condition (12 APs) and IbTX treatment (19 APs). There were no significant changes in the amplitude \[ t(29) = 1.00, P = 0.33, \text{unpaired Student’s } t\text{-test} \] or the duration \[ \text{APD}_{50}, \text{measured at 50\% of spike amplitude: } t(29) = 0.74, P = 0.47, \text{unpaired Student’s } t\text{-test} \] of APs (Fig. 9D). In contrast, the firing frequency was increased \(~25\% by IbTX \[ t(16) = 2.00, P = 0.063, \text{unpaired Student’s } t\text{-test} \]. Although the change in firing frequency was not significant, the small \( P \) value suggests a possible effect of IbTX on repetitive firing activated by depolarization in vomeronasal neurons.

Because urine-elicited responses might be different from the depolarization-induced responses, we examined the effect of IbTX on urine-induced responses. Some of the vomeronasal neurons were spontaneously active but because they did not seem to have a regular firing pattern, these cells were excluded from the analyses, although they did show increased APs in the presence of urine. Only those vomeronasal neurons that were quiescent in Ringer (Fig. 10A, top) and responded to 1:200-diluted urine (Fig. 10A, bottom) were counted. By comparing the urine-elicited firings of the same cells in both Ringer and 100 nM IbTX, we found that IbTX dramatically increased firing frequency (Fig. 10B, 5 cells from 5 different mice). Similar to the current-activated firing, IbTX shortened the after-potential hyperpolarization and increased the firing frequency (Fig. 10B). Kinetic analyses were performed on the urine-activated APs (Fig. 10C, 10 urine-induced APs and 28 urine + IbTX APs). Consistent with the data on current-activated APs (Fig. 9D), there was no significant effect of IbTX on the amplitude \[ t(36) = 1.08, P = 0.29, \text{unpaired Student’s } t\text{-test} \].
BK channels are important in regulating neuronal firing, either promoting or inhibiting repetitive firing in different cell types. Our data strongly support two conclusions. First, urine stimulation increases AA intracellular concentrations. Second, AA activates BK current and inhibits rapid repetitive firing in vomeronasal neurons.

Using immunocytochemistry, we found BK channel expression in both the soma and dendrites with especially high expression in dendrite knobs. The dendritic knob and microvilli region are the sites of odor transduction and the second-messenger signaling components. Thus the BK channels are close to the odor transduction machinery and in a position to be activated by odor stimulation. Additionally, BK channels were also abundantly expressed throughout the soma, especially the apical region, where they may be coupled to L-type Ca\(^{2+}\) channels and modulate neuronal firing (Ukhanov et al. 2007).

Previous reports have shown that BK channels are sensitive to Ca\(^{2+}\). This is true in vomeronasal neurons as well, since disruption of the Ca\(^{2+}\) influx eliminated responses to AA (Fig. 4). However, we also found evidence suggesting that Ca\(^{2+}\) was not the only activation mechanism involved. At the depolarizing potentials (approximately +40 to +100 mV, where Ca\(^{2+}\) current reverses), no or little Ca\(^{2+}\) would move into the cells but AA was still able to activate BK currents, even at +100 mV (Fig. 2B). This current-to-voltage relationship was different from what was observed without AA. Ukhanov et al. (2007) showed that BK current has an N-shape current-to-voltage relationship with a peak near the Ca\(^{2+}\) reversal potential (approximately +40 mV). Therefore the AA-activated Ca\(^{2+}\) entry, although required, appears not be the only mechanism involved. Research from several groups report that AA directly activates BK channels without the need for Ca\(^{2+}\) (Ahn et al. 1994; Clarke et al. 2002; Denson et al. 2000; Sun et al. 2007). Thus both Ca\(^{2+}\) and AA may be required to achieve the optimal activation of BK channels.

The potentiation of BK currents by repetitive AA stimulation was slow to develop (Fig. 2), which suggests that such a modulatory pathway can be only activated with repetitive or prolonged odor stimulation. There are three possible explanations for the slow potentiation of BK currents. First, the diffusion of AA to the intracellular side of the membrane could be slow. AA is hydrophobic and might not easily cross to the cytoplasmic side of the membrane. Second, Ca\(^{2+}\) buildup in response to AA stimulation is slow. Although a rapid inward current induced by AA was observed in electrophysiological studies by us (Fig. 1) and others (Lucas et al. 2003; Søreh et al. 2002), our Ca\(^{2+}\) imaging data showed a slow increase in intracellular Ca\(^{2+}\) in response to repetitive stimulation with AA (Fig. 5). Third, several Ca\(^{2+}\)-binding proteins are present in vomeronasal neurons, although the relative distribution is unclear (Ino et al. 1995; Jia and Halpern 2003; Malz et al. 2000). We propose that these Ca\(^{2+}\)-binding proteins play a role in slowing intracellular Ca\(^{2+}\) buildup and reducing BK activation by AA during a single brief odor exposure. However, with repetitive stimulation, these Ca\(^{2+}\)-binding proteins saturate and BK channels are activated. One or a combination of these mechanisms may help to prevent odor adaptation from being activated on initial exposure to odors.

In summary, we confirmed the presence of BK channels in vomeronasal neurons with immunohistochemical techniques and showed that they are present in the dendritic knob. We found that urine stimulation activated BK channels, which prevented the rapid, repetitive firing of action potentials. In addition we showed that BK channel activation was mediated by the production of AA during an odor stimulation.

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