Neuronal Chloride Accumulation in Olfactory Epithelium of Mice Lacking NKCC1

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Submitted 12 September 2005; accepted in final form 28 November 2005

Nickell, William T., Nancy K. Kleene, Robert C. Gesteland, and Steven J. Kleene. Neuronal chloride accumulation in olfactory epithelium of mice lacking NKCC1. J Neurophysiol 95: 2003–2006, 2006. First published November 30, 2005; doi:10.1152/jn.00962.2005. When stimulated with odors, olfactory receptor neurons (ORNs) produce a depolarizing receptor current. In isolated ORNs, much of this current is caused by an efflux of Cl–. This implies that the neurons have one or more mechanisms for accumulating cytoplasmic Cl– at rest. Whether odors activate an efflux of Cl– in intact olfactory epithelium, where the ionic environment is poorly characterized, has not been previously determined. In mouse olfactory epithelium, we found that >80% of the summated electrical response to odors is blocked by niflumic acid or flufenamic acid, each of which inhibits Ca2+-activated Cl– channels in ORNs. This indicates that ORNs accumulate Cl– in situ. Recent evidence has shown that NKCC1, a Na+/K+/2Cl– cotransporter, contributes to Cl– accumulation in mammalian ORNs. However, we find that the epithelial response to odors is only reduced by 39% in mice carrying a null mutation in Nkcc1. As in the wild-type, most of the response is blocked by niflumic acid or flufenamic acid, indicating that the underlying current is carried by Cl–. We conclude that ORNs effectively accumulate Cl– in situ even in the absence of NKCC1. The Cl–-transport mechanism underlying this accumulation has not yet been identified.

INTRODUCTION

In most vertebrates, transduction of an odor stimulus into a receptor current occurs on the cilia of the olfactory receptor neurons (ORNs). A Ca2+-activated Cl– channel plays a central role in this transduction. Binding of an odorant to the ciliary membrane of an olfactory receptor neuron activates a G protein–coupled cascade that activates an adenylate cyclase (reviewed in Frings 2001; Schild and Restrepo 1998). The resulting cAMP gates the first of two transducing channels, a cyclic-nucleotide–gated (CNG) channel (Nakamura and Gold 1987). Ca2+ entering through the CNG channels gates the second of the transducing channels, a Ca2+-activated Cl– channel (Kleene 1993; Kurahashi and Yau 1993; Lowe and Gold 1993). It is now established that the odor-activated Cl– flux is usually outward in isolated ORNs held at resting potential (Kurahashi and Yau 1993; Zhanazarkov and Ache 1995). In newts, this amplifying Cl– current can be as much as 85% of the receptor current (Lowe and Gold 1993). Another study (Reisert et al. 2003) suggests that >80% of the receptor current in rat may be carried by Cl–.

To support such a Cl– efflux, the neurons must accumulate cytoplasmic Cl– to concentrations that cannot be accounted for by passive equilibration through Cl– leak channels. Estimates of cytoplasmic Cl– concentration with Cl–-sensitive fluorescent dyes (Kaneko et al. 2001, 2004; Nakamura et al. 1997) and energy-dispersive X-ray microanalysis (Reuter et al. 1998) also suggest accumulation of Cl–. Typically Cl– accumulation is caused by transporters or exchangers that allow coupled fluxes of Cl– and other ions to cross the plasma membrane. Many neurons accumulate Cl– through the activity of NKCC1, a cation-coupled Cl– cotransporter (Russell 2000). NKCC1 allows the unidirectional cotransport of one Na+, one K+, and two Cl– ions across the membrane (Russell 2000). Two recent reports have identified NKCC1 as a transporter that contributes to Cl– accumulation in ORNs. In the first (Kaneko et al. 2004), a Cl–-sensitive fluorescent dye was used to monitor the cytoplasmic Cl– concentration ([Cl–]c) at the apical end of the ORNs in intact epithelium. NKCC1-mediated Cl– accumulation should be reduced by treating the epithelium with Na+-free Ringer or with bumetanide, a blocker of NKCC1. Each of these treatments reduced [Cl–]c. In the second report (Reisert et al. 2005), it was shown that most ORNs in mouse express NKCC1. Treatment with bumetanide greatly reduced the neuronal response of isolated mouse ORNs to odors. In addition, the odor response was reduced about sevenfold in ORNs from mice deficient in NKCC1. The Cl– component of the remaining receptor current was smaller in ORNs from the Nkcc1–/– mice, again indicating that NKCC1 is required for normal accumulation of Cl–.

These results show that NKCC1 plays an important role in Cl– accumulation in ORNs. However, it is unclear whether isolated ORNs are a good model for Cl– transport in intact olfactory epithelium. In situ, the ORN is bathed by at least two fluids: a mucus at the apical end and an interstitial fluid surrounding the dendrite and soma. The ionic concentrations of these compartments are unknown. Transporters that cause Cl– to accumulate in isolated ORNs might function differently if ionic concentrations in situ differ from those used when studying isolated cells. In addition, epithelial cells typically use multiple transporters to regulate cytoplasmic Cl– and other ions. These transport functions are often strongly interdependent. Thus it is probable that NKCC1 is one part of a larger system that may function differently in intact tissue than in isolated cells.

These considerations suggest two important questions. First (and surprisingly), the contribution of the Cl– current to the olfactory response in intact tissue has never been determined. Second, the contribution of NKCC1 in intact tissue is unknown. To address these questions, we studied the EOG in
intact mouse olfactory epithelium. The EOG is an extracellular field potential measured at the surface of the epithelium in response to odor stimulation. It arises from the summed activities of many ORNs near the recording electrode (Ottoson 1956; Scott and Scott-Johnson 2002). Before the mechanisms of olfactory transduction were identified in isolated ORNs, the ionic basis of the EOG had been extensively studied (reviewed by Ishimaru 1992; Leveau et al. 1989). Whether Cl− might contribute to the EOG was never decided (Takagi et al. 1966, 1968). We now report that the EOG in wild-type mice is primarily caused by a depolarizing Cl− current. Surprisingly, we found that olfactory epithelium from mice lacking NKCC1 also supports a large Cl− efflux on odor stimulation. This evidence indicates that much of the neuronal Cl− accumulation in intact olfactory epithelium is not accounted for by NKCC1 activity.

METHODS

The electroolfactogram (EOG) is a summated receptor potential activated by odors and measured at the surface of the olfactory epithelium (Ottoson 1956; Scott and Scott-Johnson 2002). The EOG was recorded while rinsing the epithelium with a thin layer of Ringer as described by Chen et al. (2000). This allows reversible changes of the solution bathing the mucosa.

EOGs were recorded in epithelia from Nkcc1+/+ and Nkcc1−/− mice in an inbred FVBN background. The Nkcc1−/− mice have a null allele for Nkcc1 (Flagella et al. 1999). NKCC1 homozygous mutant and wild-type mice were obtained by breeding gene-targeted NKCC1 heterozygous mutant mice. The genotype of each mouse was determined by a PCR of DNA from tail biopsies as described previously (Flagella et al. 1999). Nkcc1−/− mice exhibit defects in hearing, balance, salivation, blood pressure, and spermatogenesis (reviewed in Delpire and Mount 2002). Their olfactory behavior has not been reported. The genotype of the mouse was not revealed to the person recording the EOGs until the experiment had been completed. The mice were 19–52 days old.

For each experiment, a mouse was asphyxiated with CO2 and decapitated in accordance with institutional and NIH guidelines. The head was hemisected in a midsagittal plane with the blade passing between the septum and the lateral mucosa. The septum was removed, and recordings were made from the olfactory turbinate, which are located on the lateral mucosa. Most EOGs were recorded from endoturbinate III (using the nomenclature of Ressler et al. 1993). The Ringer consisted of (in mM) 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, adjusted to pH 7.2 with NaOH.

The half-head containing the olfactory turbinate was attached to a recording chamber and continuously perfused with oxygenated Ringer at a rate of ~250 μl/min. Perfusion solutions could be changed manually in a few seconds without a change in flow rate. Small wicks cut from laboratory tissue were placed and adjusted to provide a thin film of Ringer over the entire epithelium. A mixture of 10 odorants [2-heptanone, (S)-(+)-carvone, isoamyl acetate, anisole, pyridine, benzaldehyde, n-hexanoic acid, cineole, n-butanol, and ethyl n-butyrate (100 mM each in Ringer)] was used as stimulus. The stimulus was delivered by computer-controlled pressure ejection from a pipette attached to a manipulator and positioned upstream of the recording site. The stimulus solution was colored with fast green dye (0.03% wt/vol), allowing delivery and removal of the stimulus to be monitored. With the background flow rate used, odorant exposure approximated the duration of the ejection pressure pulse, which was 200 ms.

The recording electrode was a 20-μm-diam, fire-polished pipette pulled from hematocrit tubing and filled with Ringer. The recording pipette was attached to a stable manipulator, and the tip was positioned a few micrometers above the surface of the epithelium, as determined by an increase in electrical resistance on touching the epithelium. Electrical signals were amplified by a high-impedance preamplifier (AK-47LN, Metametrics, Cambridge, MA), filtered at 500 Hz, and digitized at 2 kHz. Data acquisition and stimulus control were handled by a data-acquisition board (PCI-6024E, National Instruments, Austin, TX) run by Igor Pro 4 software (Wavemetrics, Portland, OR). The preparation was grounded through a 3 M KCl salt bridge. Recordings were done at room temperature (22–25°C).

The EOG was measured three times. The first (control) EOG was measured as the epithelium was perfused with normal Ringer. Then the epithelium was perfused for 5–10 min with Ringer containing 300 μM niflumic acid or flufenamic acid. These are inhibitors of the olfactory Ca2+-activated Cl− channel (Kleene 1993; Lowe and Gold 1993; Zhainazarov and Ache 1995). At the end of this perfusion, a second EOG was recorded. Finally, the epithelium was again perfused with Ringer lacking the inhibitor for 15–30 min to allow recovery from the inhibition. After this, a third and final EOG was recorded. In all but one mouse, the series of three EOGs was repeated in one or two additional locations on the epithelium.

Odorants, niflumic acid, and flufenamic acid were from Sigma-Aldrich (St. Louis, MO). Data are presented as means ± SE.

RESULTS

On stimulation with odors, isolated ORNs generate a receptor current, of which a substantial part is carried by Cl− (Kurahashi and Yau 1993; Zhainazarov and Ache 1995). To determine if this Cl− current is significant in intact tissue, we studied the EOG in mouse olfactory epithelium. The EOG is a summated receptor potential activated by odors and measured at the surface of the olfactory epithelium (Ottoson 1956; Scott and Scott-Johnson 2002). We examined the effects of niflumic acid and flufenamic acid on the EOG. Each of these is an inhibitor of the Ca2+-activated Cl− channels that are involved in olfactory transduction (Kleene 1993; Lowe and Gold 1993; Reisert et al. 2003; Zhainazarov and Ache 1995).

In olfactory epithelium from wild-type (Nkcc1+/+) mice, stimulation with a mixture of odorants produced the expected negative-going EOG (Fig. 1, left, control). On average, the amplitude of this EOG was 438 ± 65 μV (n = 7). After
perfusion with Ringer containing 300 μM niflumic acid, the amplitude of the EOG was greatly reduced (Fig. 1, left, +NFA). On average, the amplitude was 18% of the control value (Table 1). This reduction was largely reversible. After reperfusion with Ringer lacking niflumic acid, the EOG recovered on average to 71% of its original amplitude (Fig. 1, left, recovery; Table 1). Flufenamic acid (300 μM) was tested in epithelia from two wild-type mice. In one, flufenamic acid reduced the EOG amplitude to 14%. The amplitude returned to 56% of the control value after removing the inhibitor. In epithelium from a second wild-type mouse, flufenamic acid completely and irreversibly eliminated the EOG. Perfusion with Ringer lacking inhibitors reduced the amplitude of the EOG by just 6 ± 8% (n = 4) over 30 min. The results indicate that the olfactory receptor potential in intact olfactory epithelium is primarily caused by an outward (depolarizing) flow of Cl⁻.

In mice lacking the NKCC1 cotransporter (Nkcc1⁻/⁻; Fig. 1, right, control), the mean amplitude of the EOG was 268 ± 19 μV (n = 7), which was 39% smaller than the mean determined in wild-type mice (P < 0.05). As in wild-type mice, niflumic acid greatly reduced the amplitude of the EOG (Fig. 1, right, +NFA). On average, the amplitude was reduced to 17% of the control value (Table 1). The amplitude of the EOG mostly recovered (to 68% of the control value) after removal of niflumic acid (Fig. 1, right column, recovery; Table 1). Flufenamic acid decreased the EOG amplitude to 20% of the control value in the only Nkcc1⁻/⁻ olfactory epithelium tested; the amplitude returned to 57% after removal of the inhibitor. There were no major differences in the time courses of the EOGs in Nkcc1⁺/⁺ and Nkcc1⁻/⁻ mice.

**Discussion**

We report that the EOG in mice is primarily caused by a Cl⁻ current. The amplitude of the EOG was reversibly reduced by 82% after perfusing the tissue with niflumic acid (Fig. 1, Table 1). Flufenamic acid was also effective. These reagents have been shown to block the Ca²⁺-activated Cl⁻ channels that are present in olfactory cilia, where transduction occurs. They do not block the cyclic-nucleotide-gated cationic channels that also contribute to transduction (Kleene 1993). In isolated frog ORNs, the concentration of niflumic acid used (300 μM) blocks 90% of the Cl⁻ channel current (Kleene 1993). Thus it is possible that the fraction of the receptor current caused by Cl⁻ in situ is greater than the 82% that was measured.

**Table 1. Reduction of the EOG by niflumic acid in Nkcc1⁺/⁺ and Nkcc1⁻/⁻ mice**

<table>
<thead>
<tr>
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<th>Control</th>
<th>Niflumic Acid</th>
<th>Recovery</th>
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<tr>
<td>Nkcc1⁺/⁺ (n = 5)</td>
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<tr>
<td>Amplitude, μV</td>
<td>362 ± 58</td>
<td>67 ± 10</td>
<td>258 ± 50</td>
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<tr>
<td>Percent control</td>
<td>(100)</td>
<td>18 ± 2</td>
<td>71 ± 9</td>
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<tr>
<td>Nkcc1⁻/⁻ (n = 6)</td>
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<td></td>
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<tr>
<td>Amplitude, μV</td>
<td>270 ± 23</td>
<td>44 ± 12</td>
<td>174 ± 22</td>
</tr>
<tr>
<td>Percent control</td>
<td>(100)</td>
<td>17 ± 4</td>
<td>68 ± 6</td>
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Values are ± SE. EOGs were recorded in series under three conditions (tissue in Ringer, then Ringer with 300 μM niflumic acid, then again in Ringer). Each column of the table represents one of the EOGs. In all but one mouse, the entire series was performed two or three times. For each mouse, a single average value was calculated for each EOG so that each animal was weighted equally in the table shown. EOG, electroolfactogram.

Recent evidence indicates that the cation-coupled Cl⁻ cotransporter NKCC1 underlies the accumulation of Cl⁻ by ORNs (Kaneko et al. 2004; Reisert et al. 2005). We find that the amplitude of the EOG in situ is reduced by just 39% in mice lacking NKCC1. In both strains, blockers of the Ca²⁺-activated Cl⁻ channels reduced the amplitude by >80%. There were no obvious differences in the time-courses of the EOGs. Thus it is clear that ORNs can effectively accumulate Cl⁻ even in the absence of NKCC1.

In fact, there were already three lines of evidence that NKCC1 activity is not sufficient to fully account for Cl⁻ accumulation by ORNs. 1) In rat, the concentrations of Na⁺, K⁺, and Cl⁻ on both sides of the distal dendrite were measured by energy-dispersive X-ray microanalysis (Reuter et al. 1998). These concentrations suggest that NKCC1 could not support apical Cl⁻ accumulation. Lowering [Na⁺]ₐ from the measured value of 53 ± 31 (Reuter et al. 1998) to <23 mM would allow Cl⁻ accumulation (Kaneko et al. 2004). 2) Treating the epithelium with Na⁺-free Ringer or bumetanide reduced apical [Cl⁻]ₐ in ORNs. However, [Cl⁻]ₐ reached a steady-state level of ~40 mM after these treatments, compared with ~54 mM before treatment (Fig. 5B of Kaneko et al. 2004). As the authors mention, passive equilibration of Cl⁻ would cause [Cl⁻]ₐ to be <10 mM. In other words, accumulation of Cl⁻ is robust even after treatments designed to block NKCC1. 3) In isolated mouse ORNs treated with bumetanide, niflumic acid still reduced the odor-activated current by ~30% (Reisert et al. 2005). Again, this suggests that the neurons accumulate Cl⁻ even when NKCC1 is blocked.

Our studies indicate that the EOG consists primarily of a Cl⁻ current, even in mice lacking NKCC1. These conclusions rest on two premises. First, it is generally believed that the EOG arises from the receptor potentials of the ORNs with little direct contribution from other epithelial cells (Ottoson 1956; Scott and Scott-Johnson 2002). Extensive evidence supports this view. Transection of the olfactory nerve reduces the number of ORNs, and the amplitude of the EOG decreases in parallel (Takagi and Yajima 1965). The EOG is virtually eliminated in mice lacking the CNGA2 subunit of the CNG channel (Brunet et al. 1996) or the type III adenylate cyclase (Wong et al. 2000). These transduction proteins are expressed in ORNs but not in other cells of the olfactory epithelium (Bakalyar and Reed 1990; Dhallan et al. 2000). Second, it is believed that niflumic acid reduces the EOG by blocking a neuronal Cl⁻ channel. These reagents block the Ca²⁺-activated Cl⁻ channels in the cilia of ORNs (Kleene 1993). The channels open during the odor response in isolated ORNs in amphibians (Kurahashi and Yau 1993; Lowe and Gold 1993; Zhainazarov and Ache 1995) and in mammals (Lowe and Gold 1993; Reisert et al. 2005). It is formally possible that niflumic and flufenamic acids might block some cationic channel in the ORNs that accounts for most of the EOG, but no such channel has been reported to date. In fact, substantial evidence indicates that the EOG is mostly caused by a cAMP-mediated cascade (Belluscio et al. 1998; Brunet et al. 1996; Chen et al. 2000; Wong et al. 2000). In this cascade, only the ciliary Ca²⁺-activated Cl⁻ channels are blocked by niflumic and flufenamic acids (Kleene 1993).

In neonatal mice, sustentacular cells of the epithelium express a leak channel that is blocked by niflumic acid (Vogalis et al. 2005). This raises the possibility that niflumic and...
flufenamic acids could reduce the amplitude of the EOG by two mechanisms. The first mechanism is a direct block of the neuronal Cl\(^-\) channels that underlie transduction. However, the blockers might also reduce neuronal Cl\(^-\) accumulation by an action on sustentacular cells. If such an action were to reduce the concentration of Cl\(^-\) bathing the ORNs, for example, this could indirectly reduce the neuronal Cl\(^-\) accumulation.

Some questions remain. First, it is not yet understood why isolated neurons from Nkcc1\(^{-/-}\) mice respond weakly to odors, whereas the intact epithelium gives a robust response. The ionic environment may be very different in situ, and there is evidence that Cl\(^-\) transport is less effective in isolated ORNs (Kaneko et al. 2004). Second, the mechanism of Cl\(^-\) accumulation in the Nkcc1\(^{-/-}\) mice is not yet known. NCC, another cation-coupled Cl\(^-\) cotransporter that often underlies Cl\(^-\) accumulation, has been detected in ORNs by PCR (Kaneko et al. 2004). Any Cl\(^-\)/HCO\(_3\)^ exchange present would support Cl\(^-\) accumulation. ATP-driven Cl\(^-\) pumps could also exist, but evidence for these is very limited. In Nkcc1\(^{-/-}\) mice, other mechanisms of Cl\(^-\) accumulation may be upregulated compared with the wild-type. It is not clear how multiple mechanisms might work together in the wild-type. Having multiple methods of Cl\(^-\) accumulation available should allow this function to persist despite changes in the ionic environment. A similar argument may explain why both cationic and Cl\(^-\) currents are used to depolarize ORNs (Kleene and Pun 1996; Kurahashi and Yau 1993).

ACKNOWLEDGMENTS
We thank G. Shull for providing the mice and for a critical review of the manuscript.

GRANTS
This work was supported by National Institute on Deafness and Other Communication Disorders Grants DC-00926 and DC-04203.

REFERENCES