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Neuronal chloride accumulation in olfactory epithelium of mice lacking NKCC1

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Abstract

When stimulated with odorants, olfactory receptor neurons (ORNs) produce a depolarizing receptor current. In isolated ORNs, much of this current is due to 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 find that >80% of the summated electrical response to odors is blocked by niflumic acid or flufenamic acid, each of which inhibits Ca^{2+} -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 Ca^{2+} -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 Schild and Restrepo 1998; Frings 2001). The resulting cAMP gates the first of two transducing channels, a cyclic-nucleotide-gated (CNG) channel (Nakamura and Gold 1987). Ca^{2+} entering through the CNG channels then gates the second of the transducing channels, a Ca^{2+} -activated Cl^- channel (Kurahashi and Yau 1993; Kleene 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; Zhainazarov and Ache 1995). In newt, 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 (Nakamura et al. 1997; Kaneko et al. 2001, 2004) and energy-dispersive X-ray microanalysis (Reuter et al. 1998) also

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suggest accumulation of Cl^- . Typically Cl^- accumulation is due to 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 accumulation of Cl^- by mammalian ORNs. In the first (Kaneko et al. 2004), a Cl^- -sensitive fluorescent dye was used to monitor the cytoplasmic Cl^- concentration ($[\text{Cl}^-]_{\text{in}}$) 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 $[\text{Cl}^-]_{\text{in}}$. 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 7-fold 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 demonstrate 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 which 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 summated 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 Levetau et al. 1989; Ishimaru 1992). Whether Cl^- might contribute to the EOG was never decided (Takagi et al. 1966, 1968). We now report that the EOG in wild-type mouse is primarily due to 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 polymerase chain reaction (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 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 to 52 days old.

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

The half-head containing the olfactory turbinates 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 ten odorants (2-heptanone, (S)-(+)-carvone, isoamyl acetate, anisole, pyridine, benzaldehyde, *n*-hexanoic acid, cineole, *n*-butanol, and ethyl *n*-butyrate, 100 µM 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% w/v), 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-diameter, 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 µm above the surface of the epithelium, as determined by an increase in electrical resistance upon touching the epithelium. Electrical signals were amplified by a high-impedance preamplifier (Metametrics AK-47LN, 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 to 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 to 30 min to allow recovery from the inhibition. Following 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 mean ± SEM.

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 electroolfactogram (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 Ca^{2+} -activated Cl^- channels that are involved in olfactory transduction (Kleene 1993; Lowe and Gold 1993; Zhainazarov and Ache 1995; Reisert et al. 2003).

In olfactory epithelium from wild-type (*Nkcc1*^{+/+}) mice, stimulation with a mixture of odorants produced the expected negative-going EOG (Fig. 1, left column, “control”). On average, the amplitude of this EOG was $438 \pm 65 \mu\text{V}$ ($n = 7$). Following perfusion with Ringer containing $300 \mu\text{M}$ niflumic acid, the amplitude of the EOG was greatly reduced (Fig. 1, left column, “+NFA”). On average, the amplitude was 18% of the control value (Table 1). This reduction was largely reversible. Following reperfusion with Ringer lacking niflumic acid, the EOG recovered on average to 71% of its original amplitude (Fig. 1, left column, “recovery”; Table 1). Flufenamic acid ($300 \mu\text{M}$) 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 $6 \pm 8 \%$ ($n = 4$) over 30 min. The results indicate that the olfactory receptor potential in intact olfactory epithelium is primarily due to an outward (depolarizing) flow of Cl^- .

In mice lacking the NKCC1 cotransporter (*Nkcc1*^{-/-}; Fig. 1, right column, “control”), the mean amplitude of the EOG was $268 \pm 19 \mu\text{V}$ ($n = 7$), 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 column, “+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 reduced 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 mouse is primarily due to 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 Ca^{2+} -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 ($300 \mu\text{M}$) blocks 90% of the Cl^- channel current (Kleene 1993). Thus it is possible that the fraction of the receptor current due to Cl^- *in situ* is greater than the 82% that was measured.

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 Ca^{2+} -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 $[\text{Na}^+]_{\text{in}}$ from the measured value of 53 ± 31 mM (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 $[\text{Cl}^-]_{\text{in}}$ in ORNs. However, $[\text{Cl}^-]_{\text{in}}$ reached a steady-state level of ~ 40 mM following these treatments, compared to ~ 54 mM prior to treatment (Fig. 5B of Kaneko et al. 2004). As the authors mention, passive equilibration of Cl^- would cause $[\text{Cl}^-]_{\text{in}}$ 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 $\sim 30\%$ (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., 1990).

Second, it is believed that niflumic and flufenamic acids reduce the EOG by blocking a neuronal Cl^- channel. These reagents block the Ca^{2+} -activated Cl^- channels in the cilia of frog 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 due to a cAMP-mediated cascade (Brunet et al. 1996; Belluscio et al. 1998; Wong et al. 2000; Chen et al. 2000). In this cascade, only the ciliary Ca^{2+} -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, then 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 odorants, while 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 $\text{Cl}^-/\text{HCO}_3^-$ exchangers 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 to 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 (Kurahashi and Yau 1993; Kleene and Pun 1995).

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FIG. 1.

Reduction of the EOG by niflumic acid in *Nkcc1*^{+/+} and *Nkcc1*^{-/-} mice. For each recording, a mixture of 10 odorants was applied for 200 ms onto the lateral turbinate of the olfactory epithelium of a mouse. Mice were *Nkcc1*^{+/+} (left column) or *Nkcc1*^{-/-} (right column). EOGs were first recorded while the epithelium was perfused with mammalian Ringer ("control"); these are shown at the top. Then the EOG was recorded again following treatment with Ringer plus 300 μ M niflumic acid ("NFA", middle row). Finally, the EOG was recorded after reperfusion with Ringer lacking niflumic acid ("recovery", recordings at the bottom). Further details are provided in Materials and Methods. The top traces indicate when the stimuli were given.

TABLE 1Reduction of the EOG by niflumic acid in $Nkcc1^{+/+}$ and $Nkcc1^{-/-}$ mice

	Control	Niflumic acid	Recovery
<i>Nkcc1</i> ^{+/+} (n = 5)			
Amplitude (μV)	362 ± 58	67 ± 10	258 ± 50
% control	(100)	18 ± 2	71 ± 9
<i>Nkcc1</i> ^{-/-} (n = 6)			
Amplitude (μV)	270 ± 23	44 ± 12	174 ± 22
% control	(100)	17 ± 4	68 ± 6

As described in Materials and Methods, 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.