Investigation of nasal CO_2 receptor transduction mechanisms in wild-type and GC-D knockout mice.

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Abstract

The main olfactory system of mice contains a small subset of olfactory sensory neurons (OSNs) that are stimulated by CO_2. The objective of this study was to record olfactory receptor responses to a range of CO_2 concentrations to further elucidate steps in the proposed CO_2 transduction pathway in mice. Electro-olfactograms (EOGs) were recorded before and after inhibiting specific steps in the CO_2 transduction pathway with topically applied inhibitors. Inhibition of extracellular carbonic anhydrase (CA) did not significantly affect EOG responses to CO_2, but did decrease EOG responses to several control odorants. Inhibition of intracellular CA or cyclic nucleotide-gated channels attenuated EOG responses to CO_2, confirming the role of these components in CO_2 sensing in mice. We also show that, like canonical OSNs, CO_2-sensitive OSNs depend on Ca^{2+} activated Cl^- channels for depolarization of receptor neurons. Lastly, we found that guanylyl cyclase-D knockout mice were still able to respond to CO_2 indicating that other pathways may exist for the detection of low concentrations of nasal CO_2. We discuss these findings as they relate to previous studies on CO_2-sensitive ORNs in mice and other animals.

Introduction

Vertebrate nasal cavities contain several chemo-sensory systems that provide information about the presence of food, predators, prey, potential mates, and noxious stimuli. The main olfactory system contains olfactory sensory neurons (OSNs) that are stimulated by volatile odorants, which activate a cAMP transduction pathway consisting of $G\alpha_{olf}$ (Belluscio et al. 1998), type III adenylyl cyclase (Wong et al. 2000), cAMP gated cation channels (Nakamura and Gold 1987; Brunet et al. 1996), and Ca^{2+} activated Cl^- channels (Kleene 1993; Kurahashi and Yau 1993; Lowe and Gold 1993).

The main olfactory epithelium of mice also contains a small subset of multimodal OSNs that are stimulated by CO_2 (Hu et al. 2007; Guo et al. 2009; Sun et al. 2009), carbon disulfide (Munger et al. 2010), and the natriuretic peptide hormones guanylin and uroguanylin (Leinders-Zufall et al. 2007; Zufall and Munger 2010; Arakawa et al. 2013). CO_2 is a metabolic by-product of cellular respiration in plants, animals, and heterotropic bacteria and fungi. The atmosphere currently contains 0.04% CO_2 (400 parts per million by volume), although regions of the environment may reach higher CO_2 concentrations due to decomposition, fermentation or accumulation of CO_2 in burrows from animal respiration. Nasal cavities may therefore be exposed to environmental CO_2 during inspiration in addition to 3- 4% CO_2 exhaled with each expiration.

Frogs (Coates and Ballam 1990), lizards (Coates and Ballam 1987), snakes (Coates and Ballam 1989), mice (Hu et al. 2007), and rats (Coates et al. 1998; Youngentob et al. 1991; Coates 2001; Ferris et al. 2007) detect CO_2 concentrations that are well below CO_2 concentrations in the expired air. Carbonic anhydrase (CA), an enzyme that catalyzes the reversible hydration of CO_2 to HCO_3^- and H^+, is a common constituent in the detection of nasal CO_2 in these animals. The olfactory epithelia of mice (Kimoto et al. 2004; Hu et al. 2007, Paunescu et al. 2008), rats (Brown et al. 1984; Coates 2001), guinea pigs (Okamura et al. 1996; 1999), and frogs (Coates et al. 1998) comprise small subsets of OSNs that contain CA. In addition, nasal gland cells (Okamura et al. 1996; 1999; Kimoto et al. 2004), cells in the

respiratory epithelium (Okamura et al. 1996; Coates et al. 1998), and the nasal mucosa (Kimoto et al. 2004) are known to contain CA. The CA identified in the nasal mucosa and olfactory receptors of the mouse was determined to be CA isoforms II, IV, and VI (Kimoto et al. 2004; Hu et al. 2007; Paunescu et al. 2008). A brief report by Takahashi et al. (2012) indicated that CA VII is also expressed in a small subset of mouse ORNs. Using mRNA levels to assess gene expression of CA, Tarun et al. (2003) found expression of CA XII, II, VB, IV, IX, III, XIV, I, VI, VII, listed in order of relative abundance, in the nasal epithelium of humans. While several studies have shown that intracellular CA plays a role in the detection of CO_2 by OSNs (Coates et al. 1998; Ferris et al. 2007; Hu et al. 2007) the role of mucosal (extracellular) CA in CO_2 or odorant detection is not known. A few reports have suggested that CA inhibition can cause anosmia (Turgut et al. 2007), hyposmia (Doherty et al. 1997), or changes in mucosal pH and ion homeostasis (Cavaliere et al. 1996).

In addition to CA, CO_2-sensitive OSNs in mice contain guanylyl cyclase-D (GC-D), cGMP-sensitive cyclic nucleotide-gated channels (CNG) that contain the CNGA3 subunit, and phosphodiesterase 2A (PDE2A) (Fülle et al. 1995; Juilfs et al. 1997; Hu et al. 2007; Han and Luo 2010). Sensing of CO_2 is initiated when CO_2 that has diffused across the receptor membrane is catalyzed to HCO_3^- and H^+ by cytoplasmic CA. HCO_3^- generated by this reaction activates GC-D, producing the second messenger cGMP, which causes CNG channels to open allowing for the influx of Ca^{2+} and subsequent depolarization of the OSNs (Guo et al. 2009; Sun et al. 2009). It has not been established whether these CO_2-sensitive OSNs contain Ca^{2+}- activated Cl^- channels like canonical OSNs.

The objective of this study was to investigate individual components of the proposed CO_2 transduction pathway in mice using electroolfactograms (EOG), which measure field potentials from the surface of the intact olfactory epithelium. Odorants and CO_2 were delivered to the ORNs in air-phase so as to best reproduce the normal state of the olfactory epithelium. EOGs were recorded in response to CO_2 and odorants before and after blocking intracellular CA, extracellular CA, CNG channels, or Ca^{2+}- activated Cl^- channels with topically applied

inhibitors. The specific inhibitors used were: acetazolamide (AZ), a membrane permeant CA inhibitor (Maren 1977; Henry 1987), quaternary ammonium sulfanilamide (QAS), a membrane impermerant CA inhibitor (Henry 1987), L-*cis*-diltiazem (LCD), an inhibitor of CNG channels (Leinders-Zufall et al. 2007), and niflumic acid (NA), an inhibitor of Ca^{2+}- activated Cl^- channels (Kleene 1993). To determine the role of GC-D in CO_2 sensing, EOG responses to CO_2 and odorants were recorded in GC-D knockout (KO) mice. A range of CO_2 concentrations (0,1,2,4,8,12,25,50%) were used to include those at and below exhaled values (0,1,2,4%) as well as concentrations that stimulate the trigeminal system (8,12,25,50%). Odorants (amyl acetate, citralva, cyclohexanone, propyl acetate) were used as controls given that the specific steps in the transduction pathway of canonical OSNs have been well established.

Materials and Methods

Animals

Thirty seven adult female and male C57BL/6J wild-type (WT) mice (Jackson Labs) and six adult mice lacking the Gucy2d gene (Leinders-Zufall et al. 2007) were used in these experiments. Mice were housed in a temperature controlled room (21-23°C) with a 12:12 hour light:dark schedule and were provided food and water *ad libitum*. All procedures were approved by the Allegheny College Animal Research Committee.

Surgical Procedure

Mice were euthanized with an I.P. injection of sodium pentobarbital (100 mg/Kg B.W.). To expose the olfactory epithelium, the mouse was decapitated, the head was hemisectioned, and the nasal septum and its epithelium were removed to expose the medial surface of the olfactory turbinates.

CO_2 */ Odorant Delivery System*

Odorants (amyl acetate, citralva [3,7-Dimethyl-2,6-octadienenitrile], cyclohexanone, and propyl acetate), and a range of CO_2 concentrations (0,1,2,4,8,12,25,50%) were delivered using an olfactometer similar to that previously described (Coates and Ballam 1990). The olfactometer delivery nozzle was placed 1 cm from the medial surface of the turbinates at a 45 degree angle. Breathing-quality air (0% CO_2, 21% O_2, 79% N_2) and 100% CO_2, from separate tanks, were passed through columns of anhydrous calcium sulfate and activated charcoal to remove residual odorants or oils from the tanks. The deodorized air was then humidified by bubbling it through distilled water. A gas proportioner flowmeter (Cole-Parmer) was used to mix deodorized air and CO_2 to the desired CO_2 concentrations. Odorants were from 20ml of high quality odorant stock solutions placed in 100ml glass bottles. Pressure delivered to the head space above the odorant solutions was used to mix the odorant outflow (200 ml/min) with the humidified bias airflow (1000 ml/min) delivered to the olfactory epithelium throughout the duration of the experiment, via the olfactometer. An odorant was further diluted as it traveled from the end of the olfactometer to the surface of the olfactory epithelium. The final concentration of the odorants

delivered to the olfactory epithelium, at the level of the ORNs, was not known. However, by using flowmeters, we kept the odorant concentrations constant throughout the experiment so that EOG responses to odorants could be compared before and after applying Ringers or other solutions to the olfactory epithelium. The timing and duration of odorant and CO_2 administration to the olfactory epithelium were controlled using solenoids driven by a square wave pulse generator (Grass Instruments). CO_2 concentrations were monitored using a CO_2 analyzer (BCI 9001) that had its sampling port positioned at the surface of the olfactory epithelium adjacent to the recording electrode.

Electrophysiology

EOG recording electrodes (10-15 µm tip diameter) were filled with mammalian Ringer's solution (140mM NaCl, 1.5mM KCl, 1mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM Glucose, 10 mM HEPES, pH 7.4). The recording electrode was placed in an electrode holder and positioned on the surface of the olfactory epithelium using a motorized micromanipulator (WPI, MS 314). The EOG signal was amplified (Grass DC amplifier), digitized at 100 Hz (BioPac 100) and stored on a computer for analysis using AcqKnowledge software (BioPac Inc.). The output of the square wave pulse generator as well as the CO_2 analyzer were also recorded, digitized, and stored on the computer.

Protocol

A CO_2 responsive site was located by lowering the tip of the recording electrode onto the mucosal surface of the olfactory epithelium in caudal regions of endoturbinates II and II' and delivering a two-second stimulus of 8% CO_2. If there was no EOG response to 8% CO_2, the electrode was moved to another site and the CO_2 stimulus was repeated. When a site was found that exhibited a typical negative EOG response to 8% CO_2, amyl acetate, citralva, cyclohexanone, and propyl acetate were each delivered to the olfactory epithelium in a two-second pulse to determine whether the site was also responsive to any of these test odorants. In most cases, sites that exhibited an EOG response to 8% CO_2 also exhibited EOG responses to at least three of the odorants. Next, CO_2 stimuli (0, 1, 2, 4, 8, 12, 25, 50%) were administered, in

increasing concentrations, to the epithelium to generate a baseline dose-response function. Test odorants that elicited an EOG were again delivered to the olfactory epithelium to record a baseline odorant EOG. At least 60 seconds elapsed between odorant stimuli and between CO_2 stimuli to allow time for EOG responses to recover to baseline.

After recording baseline EOG responses to odorants and CO_2, at a single site, the olfactory epithelium was treated with either: 1) Ringers, 2) 0.1 mM acetazolamide (AZ), 3) 0.1 mM quaternary ammonium sulfanilamide (QAS), 4) 0.1 mM L-*cis*-diltiazem (LCD), or 5) 0.1 mM niflumic acid (NA). All solutions were mixed in Ringers and 1% dimethyl sulfoxide (DMSO) to aid in solubility and adjusted to a pH of 7.4. The Ringers solution, used as a control, also contained DMSO. Solutions were administered to the surface of the olfactory epithelium by positioning a micropipette (tip diameter 15-20μm) next to the EOG electrode. The micropipette was connected to a pneumatic pressure pump (PicoPump), which was set to deliver a 20 nl drop of solution. Ten minutes after applying Ringers or other solutions, the range of CO_2 concentrations and odorants were delivered to the olfactory epithelium in the same order as for baseline recordings. It should be noted that the EOG responses to CO_2 and odorants were recorded at the same site and that the EOG electrode remained positioned at the site before, during, and after application of the solutions to the olfactory epithelium.

GC-D KO Mice

To determine whether CO_2 elicited EOGs in GC-D KO mice, CO_2 concentrations ranging from 0-50% were delivered in the same manner as described for WT mice. EOG responses to amyl acetate, citralva, cyclohexanone, and propyl acetate were also recorded and served as a control for the condition of the epithelium. GC-D KO mice were not treated with Ringers or any of the inhibitors.

Data Analysis

Using Acqknowlege software (BioPac, Inc.) the baseline-to-peak amplitude of the negative EOG waveform was determined for CO_2 concentrations and odorants before and after topical application of Ringers, AZ, QAS, LCD, or NA. In the few cases where CO_2 caused an

EOG with a double peak, only the amplitude of the initial peak was analyzed. To determine the effect of treatment on the EOG response amplitude at each of the CO_2 concentrations, the "Fit Model" platform of JMP 8.02 for Mac (2009 SAS Institute, Inc.) was used (Repeated Measures Two-Way ANOVA and LSMeans Tukey HSD). The analysis used a mixed model in which CO_2 concentration, treatment (baseline vs. after), and the CO_2 x treatment interaction were fixed factors and subject (a particular mouse) was considered a random effect.

Analysis of the effect of treatment on odorants was performed using a paired t-test (two-tailed) that compared the values of the baseline EOG response amplitudes to the EOG amplitudes after application of the solutions. Because the odorants used in this study exhibited large differences in EOG amplitudes, odorant responses were normalized as a percent of baseline EOG amplitudes for the purposes of graphing. Actual EOG response amplitudes, measured in mV, are given in the legend of each figure. Significant differences were defined as $P < 0.05$. Values in figures and throughout the text are reported as averages ± SEM.

Results

Sites exhibiting EOG responses to CO_2 were most often found in caudal regions of endoturbinates II and II'. An average of 20 attempts (range = 8 to 45) was needed to locate a site on the olfactory epithelium exhibiting a response to CO_2. While only negative EOG waveforms were analyzed in this study, other EOG waveform patterns were observed with CO_2 administration (Figure 1). In addition to the typical negative EOG shown in Figure 1A, some EOGs exhibited large positive waveforms following the initial negative deflection (Figure 1B,C) or contained only a positive waveform (Figure 1D). When an EOG with a large positive waveform was located, we were often able to locate a typical negative EOG by repositioning the electrode 10-50 µm laterally. EOGs recorded in response to 25 and 50% CO_2 sometimes exhibited a second negative waveform (Figure 1E, Figure 2A, Figure 3A), although this pattern was never observed for CO_2 concentration below 25% or for any of the odorants.

Fig 1

Administering Ringers to the olfactory epithelium did not significantly affect EOG responses to CO_2 or odorants (Figure 2). Figures 2A and 2B show examples of EOG responses to CO_2 and amyl acetate before (Figure 2A) and after (Figure 2B) topical application of Ringers. In this example, EOG responses to CO_2 were larger after application of Ringers, while the EOG response to amyl acetate did not appear to change. Prior to and after application of Ringers, increasing CO_2 concentrations caused an increase in EOG response amplitudes and elicited a measureable EOG response to 0% CO_2 (Figure 2A,B). The average EOG response to the range of CO_2 concentrations (Figure 2C) shows that administration of a drop of Ringers solution next to the recording electrode did not significantly affect EOG responses at any of the CO_2 concentrations. Figure 2D shows that administration of Ringers did not significantly affect EOG responses to odorants even though there appears to be a slight decrease in EOG response amplitudes after application of Ringers.

Fig 2

Topical application of 0.1mM AZ, a CA inhibitor that is membrane permeant, attenuated EOG responses to CO_2 but did not affect EOG responses to odorants (Figure 3). Figures 3A and 3B show a series of typical EOG responses to CO_2 and amyl acetate where AZ greatly reduced EOG responses to all CO_2 concentrations tested and, in this example, reduced the EOG response to amyl acetate. Figure 3C shows that application of AZ significantly attenuated average EOG responses to CO_2. Figure 3D shows that, on average, administration of AZ did not significantly affect EOG responses to any of the odorants tested.

Fig 3

Administration of 0.1mM QAS, a CA inhibitor that is membrane impermeant, caused a slight attenuation of the EOG response to CO_2 and a significant decrease in the EOG responses to amyl acetate, citralva, and propyl acetate (Figure 4). The average EOG response to the range of CO_2 concentrations (Figure 4A) shows that application of QAS appeared to attenuate the EOGs for each of the CO_2 concentrations tested, although the effect of QAS did not reach statistical significance. Figure 4B shows that administration of QAS significantly attenuated the EOG responses to the propyl acetate, amyl acetate, and citralva but did not significantly affect cyclohexanone.

Fig 4

Topical application of LCD, an inhibitor of cGMP-sensitive CNG channels, reduced EOG responses to CO_2 and odorants (Figure 5). Figures 5A and 5B show an example where administration of LCD attenuated both the EOG responses to CO_2 and propyl acetate. Averaged results show that EOG responses to higher concentrations of CO_2 (Figure 5C) and odorants (Figure 5D) were significantly attenuated by the application of LCD.

Fig 5

Inhibition of Ca^{2+}activated Cl^- channels with topical application of 0.1 mM niflumic acid caused a large decrease in EOG response amplitudes for both CO_2 and odorants (Figure 6). Figures 6A and 6B show an example where application of NA greatly attenuated, but did not eliminate, EOG responses to CO_2 and citralva. On average, treatment of the olfactory epithelium with NA caused a significant decrease in EOG responses to CO_2 (Figure 6C) and the odorants, propyl acetate, citralva, and cyclohexanone (Figure 6D). The average EOG response to amyl acetate also appeared to be attenuated after the application of NA, but this effect did not reach statistical significance.

Fig 6

A comparison between the average baseline EOG responses to CO_2 in WT mice, used in the previous experiments, and six GC-D KO mice shows that GC-D KO mice exhibited a dose-response relationship similar to that of WT mice (Figure 7A). Because the GC-D KO mice were developed from a mixed background of WT mice, a direct statistical comparison of olfactory responses to CO_2 and odorants of WT and GC-D KO mice is not appropriate. However, results show that the six GC-D mice exhibited significant ($P < 0.0001$, Repeated Measures ANOVA) EOG responses to increasing CO_2 concentrations (Figure 7A), although the EOG amplitudes appeared to be smaller than the EOG responses to CO_2 recorded in WT mice. The average EOG responses to odorants in GC-D KO mice appeared to be similar to that in WT mice (Figure 7B).

Fig 7

Discussion

The main objective of this study was to record olfactory receptor responses to odorants and a range of CO_2 concentrations to further elucidate steps in the proposed CO_2 transduction pathway in mice. We found that inhibition of extracellular CA did not greatly affect olfactory receptor responses to CO_2, but did significantly attenuate olfactory receptor responses to some of the odorants tested. Inhibition of intracellular CA or CNG channels attenuated EOG responses to CO_2, which confirms the role of these components in CO_2 sensing in mice. We also show that, like canonical OSNs, CO_2-sensitive OSNs depend on Ca^{2+} activated Cl^- channels for depolarization of the receptor neurons. Lastly, we found that GC-D knockout mice were still able to respond to CO_2 indicating that other pathways may exist for the detection of low concentrations of nasal CO_2. We discuss these findings as they relate to previous studies on CO_2-sensitive ORNs in mice and other animals.

Location of CO_2*-Sensitive Sites and Shape of EOG Waveforms*

Sites exhibiting an EOG response to CO_2 were most often found in dorso-caudal regions of endoturbinates II and II', which are areas with a relatively high density of CA-positive OSNs (Brown et al. 1984; Coates 2001; Kimoto et al. 2004; Hu et al. 2007). Even with knowledge of CA distribution, it required an average of 20 attempts and 15 minutes to locate sites on the olfactory epithelium exhibiting a typical negative EOG response to 8% CO_2. Only negative EOG waveforms were analyzed in this study (Figure 1A). While it's well established that negative EOG waveforms represent depolarization of a small population of olfactory receptors, as measured in the nasal mucosa above the receptors (Scott and Scott-Johnson 2002), the origin of positive waveforms recorded from the olfactory epithelium is not known. It has been speculated that positive waveforms are due to damaged tissue (Scott and Scott-Johnson 2002), glandular secretions (Okano and Takagi 1974), or recordings of currents returning from distant active sites (Ottoson and Shepherd 1967). This latter explanation is supported by our observations that when CO_2 caused positive waveforms, a negative EOG response could often be located by

moving the electrode, laterally, 10-50 μm.

High concentrations of CO_2 (25 and 50%) occasionally caused a second negative waveform during the recovery phase of the EOG (Figures 2A and 3A). We did not observe this for the odorants tested and the pattern does not appear to be the same as multiple EOG oscillations reported for high concentrations of odorants in other studies (Dorries and Kauer 2000; Suzuki et al. 2004). In those cases, high concentrations of odorants caused oscillations ranging from 3.5 to 40.0 Hz (Dorries and Kauer 2000; Suzuki et al. 2004) during the peak of the EOG. Given that we observed only one additional negative waveform during the recovery phase of the EOG, we conclude that this specific pattern was due to the long stimulus duration (2 seconds) and the relatively rapid recovery of the EOG response to CO_2. In preliminary experiments for another study, we found that stimulus durations less than one second did not cause a second negative waveform during any phase of the EOG.

Dose-Response Functions

The baseline dose-response functions (Figures 2-7) show that increasing CO_2 concentrations caused an increase in EOG amplitudes up to around 12% CO_2 at which point a response maximum was reached. In addition, in all cases, 1% CO_2 generated EOGs and in several cases, measurable EOGs were recorded in response to 0% CO_2. Because switching to a second air source with 0% CO_2 never exhibited "EOG-like" responses we conclude that there must have been residual CO_2 in the delivery nozzle at a concentration below the resolution of our respiratory CO_2 analyzer (BCI 9001). Several studies have shown that CO_2 detection thresholds in mice (Hu et al. 2007) and rats (Youngentob et al. 1991; Ferris et al. 2007) are below 1% CO_2. In the study using mice (Hu et al. 2007), behavioral detection thresholds were determined to be 0.066%, while Long-Evans rats (Youngentob et al. 1991) and Zucker rats (Ferris et al. 2007) where found to have detection thresholds of 0.52% (ranging from 0.04 to 1.7%) and 0.48% (ranging from 0.26 to 0.69%), respectively. Those results and the results reported here show that mice and rats can detect extremely low concentrations of CO_2 despite having nasal cavities

exposed to 3-4% CO_2 during each expiratory cycle (Lahiri 1975). Given the dorso-caudal location of CO_2-sensitive olfactory receptors (Brown et al. 1984; Coates 2001; Kimoto et al. 2004; Hu et al. 2007), it appears that these receptors are positioned, within the nasal cavity, to sense CO_2 during an inspiratory cycle but not an expiratory cycle (Zhao et al. 2006; Yang et al. 2007; Jiang and Zhao, 2010).

The response maximum of around 12% CO_2 that we found for mice is consistent with the response maximum observed in rats (Coates 2001) and frogs (Coates and Ballam 1990). In those studies, and the current study, we delivered CO_2 to the surface of the olfactory epithelium using similar olfactometers. Recordings from CO_2-sensitive bulbar neurons in the mouse, however, showed a response maximum around 1-2% CO_2 (Hu et al. 2007). These different response maximums are most likely due to differences in the way that EOGs and single-unit recordings represent olfactory responses to stimuli. EOGs are a measure of the summated receptor potentials of a small number of olfactory receptors and are recorded as field potentials from the surface of the olfactory epithelium. Unlike single-unit recordings, the number of receptor neurons and the extent of the field potential in EOG recordings are unknown. Therefore, larger EOG amplitudes indicate increasing concentrations of the stimulus in addition to an increased number of receptors from which responses are recorded. It is also possible that the response profiles of CO_2-sensitive neurons in the olfactory epithelium and the CO_2-sensitive neurons in the olfactory bulb are different. A study that recorded responses of individual GC-D+ OSNs to CS_2 and CO_2 (Munger et al. 2010) found that these neurons were 10,000 times less sensitive to CO_2 than CS_2 and reported a threshold response to CO_2 of 6.8mM recorded in submerged olfactory tissue. Based on the authors' report that bubbling 100% CO_2 in HEPES-buffered saline resulted in a CO_2 concentration of approximately 34mM, a concentration of 6.8mM in solution is estimated to be equivalent to 20% CO_2 delivered using the type of olfactometer in our study.

The results from the present study and those cited above illustrate that olfactory receptor response profiles to CO_2 may be dependent on the method of CO_2 delivery, making it difficult to compare results across studies. Takahashi et al. (2012) recently provided a brief report showing

that another subset of ORNs, which do not contain GC-D, CAII, and are not sensitive to urinary peptides nor CS_2, are sensitive to CO_2. Therefore, it's also possible that the different CO_2 response profiles are due to different subsets of ORNs sensitive to CO_2.

Roles of intracellular and mucosal CA in detecting nasal CO_2

Several CA isoforms have been identified in the nasal epithelium of the mouse. CA II is a cytoplasmic form found in a small subset of ORNs (Kimoto et al. 2004; Hu et al. 2007; Paunescu et al. 2008). In addition, Takahashi et al. (2012) reported that CA VII is localized to a novel subset of ORNs that appear to be sensitive to CO_2. A membrane bound isoform, CA IV, has also been found in a small population of OSNs and is co-localized with the vacuolar proton-pumping ATPase (V-ATPase) (Paunescu et al. 2008). The nasal mucosal CA isoform has been identified as CAVI, which is a secreted form of CA also found in salivary, lacrimal, and mammary glands (Fernley et al. 1989; Ogawa et al. 2002; Kimoto et al. 2004).

We found that application of AZ, a membrane permeant CA inhibitor (K_i = 10nM; Maren 1977; Henry 1987), to the surface of the olfactory epithelium significantly attenuated the olfactory receptor responses to CO_2 (Figure 3C) indicating that intracellular CA is a necessary component for the detection of nasal CO_2 in mice. These findings, along with the results from a study using CAII knockout mice (Hu et al., 2007) and our results showing that inhibition of extracellular CA with the membrane impermeant inhibitor QAS did not significantly affect the olfactory receptor responses to CO_2 (Figure 4A), clearly establish the role for intracellular CA in the transduction pathway of nasal CO_2. Similarly, other respiratory-related CO_2 chemoreceptors, such as brainstem (Coates et al. 1991; Nattie 1999), carotid body (Iturriaga et al. 1993), laryngeal (Coates et al. 1996), and trigeminal receptors (Komai and Bryant 1993; Bryant 2000) depend on intracellular CA to sense CO_2.

While the role for intracellular CA seems clear, it is not established whether CA IV and CA VI play any role in the detection of nasal CO_2 in mice. The locations of CA IV and VI in the nasal epithelium of the mouse and the presence of V-ATPase suggest that these CA isoforms

contribute to pH homeostasis in the nasal mucus layer by buffering inhaled acids or protons generated by the exhaled CO_2 (Shusterman and Avila 2003; Kimota et al. 2004, Ferris et al. 2007; Paunescu et al. 2008). We showed that inhibition of extracellular CA with QAS (K_i = 14μM; Henry 1987) did not significantly affect the olfactory receptor response to CO_2 (Figure 4A) indicating that CA IV, CA VI, or other extracellular forms of CA do not play a major role in the detection of CO_2 by olfactory receptors. However, the application of QAS did cause a slight decrease in the average EOG amplitude at each of the CO_2 concentrations tested and caused a significant decrease in the olfactory responses to amyl acetate, citralva, and propyl acetate (Figure 4B). These results support the role for CA IV and VI in mucosal pH and ion homeostasis and suggest that mucosal CA may play a role in determining the concentration of CO_2 in the nasal mucosa as well as at the level of the OSNs. In a study on humans, administration of the CA inhibitor, dichlorphenamide, caused an increase in nasal mucosa pH 30, 60, and 90 minutes following CA inhibition (Cavaliere et al. 1996) showing that extracellular CA is important for pH homeostasis.

Although there have been no studies showing a specific role for CA in odorant transduction there have been brief reports of anosmia (Turgut et al. 2007) and hyposmia (Doherty et al. 1997) as a result of CA inhibition. Those studies and the results from the present study, showing that EOG responses to amyl acetate, citralva, and propyl acetate were attenuated after mucosal CA inhibition with QAS (Figure 4), suggest that changes in mucosal pH and ion homeostasis affect the output of the OSNs or the interaction between odorants and receptors. Alternatively, CA II exhibits some esterase activity (Gould and Tawfik 2005; Nagashima and Touhara 2010) and therefore CA inhibition may have specifically affected the acetates used in our study. Although Nagashima and Touhara (2010) showed that inhibition of mucosal CA with AZ did not affect a mouse's ability to discriminate the ester acetyl isoeugenol, they did not examine amyl acetate or propyl acetate. We found that QAS caused a greater attenuation of EOG responses to odorants than AZ indicating that QAS also exhibits esterase activity or has a greater effect on pH and ion homeostasis than AZ. The latter explanation is the most plausible

given that QAS attenuated the EOG responses to not only the esters used in this study (amyl acetate and propyl acetate) but also odorants with ketone (cyclohexanone) or nitrile (citralva) functional groups.

We cannot rule out that the effects of QAS application on the ORNs response to odorants were due to non-specific actions of the inhibitor. In preliminary studies to determine the most effective dose of QAS, we found that higher concentrations (1mM) often caused inconsistent results that were difficult to interpret, which may have been due to non-specific effects. We therefore chose to use the lower concentration of 0.1mM QAS for the present study.

Role of CNG channel in detecting nasal CO_2

Canonical OSNs contain cAMP-sensitive CNG channels that when activated by cAMP, initiate an influx of Ca^{2+} and a subsequent receptor potential (Nakamura and Gold 1987; Brunet et al. 1996). In contrast, OSNs that detect CO_2 contain cGMP-sensitive CNG channels that are comprised of the CNGA3 subunit, which is expressed exclusively in CO_2-sensitive ORNs (Meyer et al. 2000).

Topical application of 0.1 mM LCD, an inhibitor of cGMP-activated Ca^{2+} channels, attenuated the EOG responses to both odorants and some of the higher concentrations of CO_2 (Figure 5). An attenuation of the OSN response to CO_2, after administration of LCD, is consistent with previous studies that tested olfactory receptor responses to CO_2 (Hu et al. 2007; Han and Luo 2010). Our results suggest that cGMP-activated Ca^{2+} channels are necessary for the detection of a large range of CO_2 concentrations, including those concentrations typically reported to stimulate trigeminal nerve endings (Bryant 2000; Wise et al. 2004). The inhibition of odorant EOG responses (Figure 5D) indicates that LCD, at the concentrations used in this study (0.1mM), affects both cAMP-sensitive CNG channels in canonical ORNs and CNG channels containing the CNGA3 subunit in CO_2-sensitive ORNs. Although we cannot rule out the involvement of novel cationic channels, our results suggest that CNG channels containing CNGA3 subunits are an important component of the CO_2 detection mechanism in mice and

support recent findings using CNGA3 KO mice (Han and Luo 2010).

Role of Ca^{2+} activated Cl^- channel in detecting nasal CO_2

As expected, the application of 0.1 mM NA, an inhibitor of Ca^{2+} activated Cl^- channels (Kleene 1993, Nickell et al. 2006), significantly attenuated the EOG response to odorants (Figure 6D). Previous studies show that an efflux of Cl^- potentiates the receptor potential in canonical OSNs (Kleene 1993; Kurahashi and Yau 1993; Lowe and Gold 1993: Nickell et al. 2006). In addition, our results are consistent with those reported by Nickell et al. (2006) who found that EOG responses to a mixture of odorants were greatly attenuated after application of NA to the olfactory epithelium of mice. However, the role of Ca^{2+} activated Cl^- channels in olfaction has been questioned recently, when it was found that Anoctamin2 (Ano2) knockout mice exhibited only a 40% reduction in EOG responses to odorants delivered in fluid-phase and exhibited no reduction in EOG responses to odorants delivered in air-phase (Billig et al. 2011). That study also reported that disruption of *Ano2* did not reduce the performance in olfactory behavioral tasks. Our results, where the application of 0.1mM NA to the olfactory epithelium reduced the EOG response to odorants delivered in air-phase do not support the findings of Billig et al. (2011). We found that inhibition of Ca^{2+} activated Cl^- channels with topical administration of NA caused a 50-77% reduction in the EOG response to propyl acetate, amyl acetate, citralva, and cyclohexanone (Figure 6D). Our results are consistent with those reported in previous studies where it was found that application of NA caused up to an 80% reduction in receptor responses to odorants (Kleene 1993, Nickell et al. 2006, Kleene 2008). It's not clear why there is a discrepancy between the results from previous studies, our present study, and those from Billig et al. (2011) but it's clear that more studies using Ano2 knockout mice are needed.

A novel finding of the present study was that application of 0.1 mM NA significantly attenuated the EOG response to CO_2 concentrations ranging from 1 to 50% (Figure 6C). Like the results for the odorants used in this study, EOG responses to CO_2 were attenuated nearly 80% after NA administration (Figure 6C), indicating that Ca^{2+}-activated Cl^- channels play a large role

in the depolarization of CO_2-sensitive ORNs. To our knowledge, there are no other reports showing evidence for the role of Ca^{2+}-activated Cl^- channels in CO_2 detection by ORNs. The EOG waveforms shown in Figure 6B illustrate that after inhibition of Ca^{2+}-activated Cl^- channels a small EOG is still present, which we conclude represents the depolarization caused by the influx of Ca^{2+} through cGMP-sensitive CNG channels. These results indicate that, like canonical ORNs, CO_2-sensitive receptors contain Ca^{2+}-activated Cl^- channels that amplify the initial cationic current.

Although NA has been reported to inhibit various TRP channels, any effect on those channels would only be seen for the higher CO_2 concentrations (e.g. above 25%) used in the present study. In addition, we recorded EOGs from the olfactory epithelium in caudal regions of endoturbinates II and II' not from regions of the respiratory epithelium where negative mucosal potentials are recorded.

Role of GC-D in detecting nasal CO_2

A comparison of the baseline EOG responses to CO_2 for the WT and GC-D KO mice shows that GC-D KO mice exhibited a dose-response relationship similar to that of WT mice (Figure 7A). Although CO_2 appeared to cause smaller EOG response amplitudes in GC-D KO mice compared to WT mice, the data clearly show that GC-D KO mice are able to detect CO_2 at each of the concentrations used in this study. While it's known that GC-D plays an important role in the detection of guanylin, uroguanylin (Leinders-Zufall et al. 2007; Zufall and Munger 2010) and CS_2 (Munger et al. 2010), our results show that GC-D does not appear to be necessary for the detection of CO_2, suggesting that other pathways exist for CO_2 sensing in mice.

As expected, we also observed that the average EOG responses to odorants in GC-D KO mice did not appear different from EOG responses to odorants in WT mice (Figure 7B). These results confirm the observations from previous studies that GC-D does not play a role in the detection of odorants by canonical ORNs.

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Figure Legends

Figure 1: Examples of EOGs recorded in response to 8% (**A**,**B**,**C**,**D**) and 50% CO_2 (**E**). Each waveform is from a different site on the olfactory epithelium and from a different mouse. Only sites exhibiting a typical EOG waveform (**A**), in response to 8% CO_2, were analyzed in the experiments.

Figure 2: Typical EOG responses to CO_2 and amyl acetate before (**A**) and after (**B**) topical application of mammalian Ringers. **C**. Average (±SEM) baseline EOG responses to CO_2 (filled circles) and EOG responses to CO_2 after application of Ringers (open circles). N=6. **D**. Average (±SEM) EOG response to propyl acetate (N=7, P=0.191, baseline=5.5±0.9mV, after Ringers=4.3±0.9mV), amyl acetate (N=7, P=0.151, baseline=6.6±2.2mV, after Ringers =4.9±1.7mV), citralva (N=6, P=0.237, baseline=3.7±1.9mV, after Ringers =2.6±1.2mV), and cyclohexanone (N=5, P=0.235, baseline=3.9±1.7mV, after Ringers =2.9±1.1mV) after application of Ringers.

Figure 3: Typical EOG responses to CO_2 and amyl acetate before (**A**) and after (**B**) topical application of the membrane permeant CA inhibitor, acetazolamide (AZ). **C**. Average (±SEM) baseline EOG responses to CO_2 (filled circles) and after application of AZ (open circles). N=7. **D**. Average (±SEM) EOG response to propyl acetate (N=6, P=0.523, baseline=5.7±1.1mV, after AZ=4.6±1.4mV), amyl acetate (N=8, P=0.134, baseline=2.6±0.4mV, after AZ =1.5±0.5mV), citralva (N=4, P=0.999, baseline=2.5±1.4mV, after AZ=2.5±1.6mV), and cyclohexanone (N=6, P=0.266, baseline=2.6±1.2mV, after AZ=1.6±0.5mV) after application of AZ. * = $P<0.05$. ** = $P<0.001$.

Figure 4: **A**. Average (±SEM) baseline EOG responses to CO_2 (filled circles) and after (open circles) topical application of the membrane impermeant CA inhibitor, quaternary ammonium sulfanilamide (QAS). N=6. **B**. Average (±SEM) EOG response to propyl acetate (N=8, P=0.005, baseline=7.6±1.3mV, after QAS=4.1±0.9mV), amyl acetate (N=6, P=0.048, baseline=5.1±1.9mV, after QAS=3.9±2.0mV), citralva (N=6, P=0.049, baseline=2.7±0.8mV, after QAS=1.9±0.8mV), and cyclohexanone (N=5, P=0.099, baseline=3.1±1.0mV, after QAS=2.2±1.0mV) after application of QAS. * = P<0.05.

Figure 5: Typical EOG responses to CO_2 and odorants before (**A**) and after (**B**) treatment with L-*cis*-diltiazem (LCD), an inhibitor of cGMP-sensitive CNG channels. **C.** Average (±SEM) baseline EOG responses to CO_2 (filled circles) and after (open circles) application of LCD. N=6. **D.** Average (±SEM) EOG response to propyl acetate (N=6, P=0.01, baseline=11.1±1.0mV, after LCD=4.1±1.2mV), amyl acetate (N=6, P=0.034, baseline=2.1±0.4mV, after LCD=0.7±0.2mV), citralva (N=6, P=0.042, baseline=4.4±1.1mV, after LCD=1.2±0.4mV), and cyclohexanone (N=6, P=0.048, baseline=1.3±0.2mV, after LCD=0.5±0.1mV) after application of LCD. * = P<0.05.

Figure 6: Typical EOG responses to CO_2 and odorants (**A**) before and (**B**) after treatment with 0.1 mM niflumic acid (NA), an inhibitor of Ca^{2+} activated Cl^- channels. **C**. Average (±SEM) baseline EOG responses to CO_2 (filled circles) and after (open circles) application of NA. N=7. **D**. Average (±SEM) EOG response to the propyl acetate (N=7, P=0.0002, baseline=11.7±1.4mV, after NA=2.6±0.5mV), amyl acetate (N=7, P=0.104, baseline=3.2±1.5mV, after NA=0.7±0.3mV), citralva (N=7, P=0.018, baseline=2.7±0.6mV, after NA=0.9±0.3mV), and cyclohexanone (N=7, P=0.012, baseline=0.8±0.2mV, after NA=0.5±0.3mV) after application of NA. * = P<0.05, ** = P<0.001.

Figure 7: EOG responses to CO_2 and odorants in wild type and GC-D KO mice. **A.** Average (±SEM) EOG responses to CO_2 of wild type (filled circles; N=32) and GC-D KO mice (open circles; N=6). The six GC-D mice exhibited significant ($P < 0.0001$, Repeated Measures ANOVA) EOG responses to CO_2. **B.** Average (± SEM) EOG responses to odorants of wild type (solid bars; propyl acetate, N=34; amyl acetate, N=34; citralva, N=29; cyclohexanone, N=29) and GC-D KO mice (open bars; N=6 for all odorants).