For almost two decades, it has been postulated that calcium-activated Cl\(^-\) channels (CaCCs) play a role in airway epithelial Cl\(^-\) secretion, but until recently, the molecular identity of the airway CaCC(s) was unknown. Recent studies have unequivocally identified TMEM16A as a glandular epithelial CaCC. We have studied the airway electrophysiology of neonatal mice homozygous for a null allele of Tmem16a (Tmem16a\(^{-/-}\)) to investigate the role of this channel in Cl\(^-\) secretion in airway surface epithelium. When compared with wild-type tracheae, the Tmem16a\(^{-/-}\) tracheae exhibited a >60% reduction in purinoceptor (UTP)-regulated CaCC activity. Other members of the Tmem16 gene family, including Tmem16f and Tmem16k, were also detected by reverse transcription-PCR in neonatal tracheal epithelium, suggesting that other family members could be considered as contributing to the small residual UTP response. TMEM16A, however, appeared to contribute little to unstimulated Cl\(^-\) secretion, contributing to the small residual UTP response. TMEM16A, how-
gate the contribution of TMEM16A to unstimulated and Ca\(^{2+}\)-regulated Cl\(^{-}\) secretion. Regions of tracheas devoid of submucosal glands were selected for study to characterize the role of TMEM16A in superficial epithelial Cl\(^{-}\) secretion. Because tracheas from Tmem16a\(^{-/-}\) mice exhibited some residual CaCC activity, mRNA analysis was performed to identify other TMEM family members that might function as CaCCs in the neonatal trachea. We also compared the tracheal bioelectric properties of neonatal CF tracheas with those of Tmem16a\(^{-/-}\) tracheas to elucidate the relative roles of each Cl\(^{-}\) channel in airway Cl\(^{-}\) secretion and mucus clearance. Finally, we compared the histology and morphology of the tracheas from Tmem16a\(^{-/-}\) and Cfr\(^{-/-}\) mice in an attempt to link defective Cl\(^{-}\) secretion to tracheal cartilaginous defects and the mode of early postnatal death characteristic of these animals.

**EXPERIMENTAL PROCEDURES**

**Tracheal Bioelectric Properties**—All pups were removed from their mothers within 12–48 h after birth and studied immediately. Both gene mutations (Tmem16a; see Ref. 5 for details) and Cfr (cfr\(^{mumunc}\)) were on a mixed strain background. There was no significant difference in the body mass among the three genotypes of the Tmem16a mice or the Cfr\(^{-/-}\) (CF) mice and their WT littermates at this early age (data not shown). For the CF line, pups \(+/+\) for CFR are considered to be WT because we have not detected phenotypic differences in airway bioelectric properties between tracheas \(+/+\) versus \(+-/\) for CFTR (data not shown). The pups for the Ussing chamber study were killed by an overdose of ketamine/xylazine, and the tracheas were immediately removed, mounted on an Ussing chamber, and studied. All preparations were bathed bilaterally in Krebs bicarbonate ringer, as reported previously (7). The tracheas were fixed to examine histologically for the presence of submucosal glands. After removal from the chambers, these small tracheal specimens were stained with tissue marking dye (Triange Biologicals, Germantown, MD). Other RNA samples (adult lung) were purchased from Ambion. RNA was analyzed for concentration and quality on the NanoDrop spectrophotometer and Agilent 2100 bioanalyzer. 200 ng of total RNA was used to synthesize cDNA using SuperScript III reverse transcriptase and the manufacturer’s protocol (Invitrogen). For adult samples, 100 ng of cDNA was used in each PCR reaction; 200 ng was used for newborn tracheal samples. Primers were ordered specifically to cross introns and are as follows (accession numbers are given in parentheses): Tmem16a (NM_178642), forward, 5'-GGGTTTGTCCACCCCTGTHTGTG-3', reverse, 5'-GAGAGGCCTGTGATTGACGAA-3'; Tmem16b (NM_153589), forward, 5'-GGCAGTTTCTCCGTACATCAT-3', reverse, 5'-AACCTCC-TGTTCAAGCTGTTG-3'; Tmem16j (NM_175344), forward, 5'-ATGGGACAACGGTTGAGCTAC-3', reverse, 5'-TCAGGTACTCTTTGGATCGGG-3'; Tmem16j (NM_178381), forward, 5'-TGAAGGATGGGTTTTTGAG-3', reverse, 5'-CCAGAGGGCCATGAAAT-3'; Tmem16k (NM_133979), forward, 5'-TGAGTTTGTGGCTATGTGAGC-3', reverse, 5'-GGCCAGGTTCGTTGAGTGTG-3'. For RT-PCR, ImmoMix (Bioline, Taunton, MA) was used according to recommended conditions with a 25-μl volume in an ABI thermocycler. Cycling conditions were as follows: 95 °C for 10 min hot start followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, ending with 7 min at 72 °C and a 4 °C hold. All RT-PCR reactions included no reverse-transcriptase controls, which were negative (not shown), and all products were cloned from positive control tissues and sequenced to confirm product identity.

**Histology**—Preparations removed from Ussing chambers were fixed to examine histologically for the presence of submucosal glands. After removal from the chambers, these small specimens were stained with tissue marking dye (Triange Biologicals) for easy visualization and then processed. For general histology, tracheas from newborn mouse pups, euthanized by an overdose of ketamine/xylazine, were fixed in situ in neutral buffered formalin, processed, and stained with Alcian blue periodic acid Schiff (AB-PAS) or hematoxylin and eosin. For histology of the CF trachea, we utilized ΔF508 CF pups (cfr\(^{tm1kth}\)). Note that the histological defects and bioelectrics of the two CFTR mutations appear identical (8).3

**Statistics**—When more than two groups were compared (Tmem16a mice), a one-way analysis of variance was used. When only two groups were being compared (CF versus WT), a Student’s t test was used. Data are shown as means ± S.E.

**RESULTS**

The tracheal bioelectric properties of newborn mice, homozygous for a null allele of Tmem16a (referred to as Tmem16a\(^{-/-}\) in this study), and their wild-type (\(+/+\)) and heterozygous (\(+-/\)) littermates are shown in Fig. 1A. There was no significant difference in the unstimulated short circuit current (\(I_{sc}\), the response to amiloride, or the residual (post-amiloride) \(I_{sc}\) among the three genotypes. However, a one-way analysis of variance revealed that the response to UTP differed significantly (\(\ast\ast\), \(p = 0.01\)) among the three groups. Although the magnitude of the UTP response in the

3 B. R. Grubb, personal communication.
Tmem16a−/− tracheas was significantly reduced when compared with the other two groups, it should be noted that a significant residual UTP response still remained in the Tmem16a−/− tracheas. The response to forskolin did not differ among the three genotypes, but the response to bumetanide was significantly reduced in the Tmem16a−/− tracheas when compared with the other two genotypes.

We considered whether the measured bioelectric signals could have originated in the murine submucosal glands. However, because no submucosal glands were identified in any tracheal sections examined histologically (18 sections from 9 individual tracheas; data not shown) after removal of the tissue from the Ussing chambers, we concluded that the signal must originate from the epithelium lining the tracheal lumen.

In an effort to develop a comprehensive understanding of the proteins involved in the residual UTP-activated response, mRNA analyses for other TMEM family members in neonatal tracheal epithelium were conducted (Fig. 1B). Of the TMEM isoforms carefully evaluated to date, mRNA from Tmem16a, Tmem16f, and Tmem16k were shown to be expressed in both proximal and distal tracheal epithelium and mesenchyme. Thus, these members are candidates for the residual CaCC activity seen in the Tmem16a−/− mice. Other family members, including Tmem16b and Tmem16j, were not detected in neonatal tissue, although Tmem16j was clearly detected in adult mouse trachea.

The tracheal bioelectric properties of newborn WT and CF pups are shown in Fig. 1C. The unstimulated Isc was significantly reduced in the CF tracheas when compared with WT tracheas (**, p ≤ 0.01), whereas the amiloride response did not differ between the two genotypes. The post-amiloride residual Isc was also significantly reduced in the CF tracheas (**, p ≤ 0.01), likely reflecting the reduced unstimulated Isc in these preparations. The forskolin response in CF tracheas was significantly reduced (**, p ≤ 0.01) when compared with WT. The response to UTP and bumetanide did not differ between the genotypes.

Both the Tmem16a−/− and the CF tracheas (Fig. 2, B and F) exhibited anatomical defects in the cartilaginous rings that appeared similar, with reduced tracheal size when compared with WT (Fig. 2, A and E). In mice (both Tmem16a−/− and CF), the cartilaginous rings on the ventral surface were discontinuous, and the epithelial layer exhibited evaginations. However, the defect was more extensive in the Tmem16a−/− tracheas, extending the entire length of the trachea, whereas in the CF tracheas, it tended to be confined to the first 3–4 tracheal rings. There was significant mucus (red-stained mucopolysaccharide) accumulation in the lumen of the Tmem16a−/− tracheas, which nearly plugged the trachea in some animals (Fig. 2D). Most of the mucus was in the lower two-thirds of the adult trachea and lung were used as positive controls for Tmem16a, Tmem16f, Tmem16j, and Tmem16k. For Tmem16b, mouse eye and brain were used because Tmem16b was not detected in adult trachea or lung (not shown). Blank = water control; MW = molecular weight marker. Approximate sizes of products are given (in bp). C, tracheal bioelectricities of WT (solid bars, n = 29) and CF (open bars, n = 31) pups. Data addings and symbols are the same as in panel A, with the exception that forskolin was added before UTP. **, p ≤ 0.01 from WT. Data are means ± S.E (error bars).
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trachea, with little in the mainstem bronchi. The smaller airways were devoid of mucus. The CF tracheas did not contain any mucus.

DISCUSSION

CaCCs have been implicated in a variety of physiological functions, including fluid secretion, olfactory transduction (9–11), and smooth muscle contraction (12). However, until recently, the molecular identity of these channels has eluded identification. TMEM16A has now been convincingly identified as a calcium-activated Cl⁻ channel (or a subunit thereof) with a unique structure but no obvious Ca²⁺ binding sites (3).

A CaCC dominates the Cl⁻ secretory response of the murine trachea, and we have previously speculated that this Cl⁻ conductance protects the CF mouse from CF-like airway disease (1, 13). CaCC has also been demonstrated to be an important functional Cl⁻ channel in CF human airway epithelia when CFTR, the cAMP-mediated Cl⁻ channel, is non-functional (14–16).

Gene expression data indicate that the TMEM16A is expressed at high levels in exocrine glands and in organs rich in glands (2). In the mouse in vivo, it has been shown that TMEM16A mediates muscarinic regulated salivary flow, as intravenous treatment of mice with TMem16a small interfering RNA significantly reduced TMem16A expression and pilocarpine-stimulated salivary flow in submandibular glands (4).

In the present investigation, we studied the function of TMem16A in the airways of neonatal pups in which the Tmem16a gene was deleted (5) and compared these data with both WT and Cftr gene targeted mice. The unstimulated I₀ reflects components of electrogenic Na⁺ absorption and Cl⁻ secretion, and this parameter did not differ among the three Tmem16a genotypes (+/+, +/-, −/−). The amiloride-sensitive electrogenic Na⁺ absorption also did not differ among the three genotypes, nor was the amiloride-insensitive residual current, an index of unstimulated Cl⁻ secretion, different between the three genotypes. Thus, we conclude that TMem16A does not contribute to unstimulated Cl⁻ secretion, nor does it exhibit a regulatory relationship to ENaC, as proposed for CFTR (17).

As a probe of regulated Cl⁻ secretion in neonatal Tmem16a−/− tracheal preparations, UTP was used as an agonist to increase intracellular calcium, which, in amiloride-pre-treated tissues, predominantly activates CaCC. We observed a significant Tmem16a gene dose effect on the magnitude of the UTP response (Fig. 1A), with Tmem16a−/− tracheas exhibiting a significantly reduced (~60%) response when compared with the WT tissue. However, because a significant UTP response persisted in the Tmem16a−/− tracheas, it is likely that a genetically distinct CaCC also plays a role in the Cl⁻ secretory response of this tissue. A role for Bestrophin 2 in contributing to CaCC in the murine airway has been suggested by RNA inhibition studies in primary mouse tracheal cells (18). By reducing Best2 mRNA levels, the authors report a significant reduction in ATP-stimulated currents to ~50% of wild-type levels. In our study, the magnitude of the forskolin-induced Cl⁻ secretory responses did not differ among the three genotypes, suggesting that TMEM16A is not cAMP-regulated. Finally, the response to bumetanide (which blocks basolateral Cl⁻ entry and thus Cl⁻ secretion) was attenuated in the Tmem16a−/− tracheas proportional to the magnitude of the UTP responses in these tissues.

For CaCC to have its postulated protective role in the CF mouse lung (1), it must be expressed in the superficial epithelium. Immunohistochemical analysis (4) suggested surface expression in distal airway epithelium. Our histologic analyses of the region of the excised neonatal tracheas assayed in Ussing chambers revealed a well differentiated superficial epithelium with an absence of glands. Molecular analyses confirmed that Tmem16a was expressed in this epithelium (Fig. 2B). Thus, these studies are the first to assign a molecular identity to a CaCC expressed in murine superficial airway epithelia.

There are several possible candidate CaCCs that mediate the residual UTP-regulated activity in Tmem16a−/− mice. For example, there are 10 identified genes in the TMEM gene family in mice and humans, but CaCC function has only been ascribed to Tmem16a and Tmem16h. Because the Tmem16a−/− mice exhibit residual UTP- (Ca²⁺-+) stimulated Cl⁻ secretion, levels of mRNA expression of other TMEM gene family members were measured in neonatal tracheas (Fig. 2B). Tmem16f and Tmem16k, but not Tmem16b or Tmem16i, were detected.
Altematively, future studies are required to assign residual CaCC function to the protein products of TEM gene family members or other genes.

As one approach to determine the relative roles of TEM16A versus CFTR in neonatal tracheal Cl⁻ secretion, we studied CF mouse tracheas also excised 12–24 h after birth. The neonatal CF tracheas exhibited a significant reduction in the unstimulated \( I_{sc} \) when compared with WT, suggesting that a portion of this Cl⁻ secretion is mediated via CFTR (\( \sim 50\% \)). As reported for adult CF mice (13), there was no difference in the amiloride response between the two genotypes. Unlike the Tmem16a/B tracheas, the tracheas from the CF pups exhibited a significant decrease in the post-amiloride residual \( I_{sc} \), supporting the notion that the post-amiloride residual \( I_{sc} \) reflects Cl⁻ secretion. Note that because a significant post-amiloride \( I_{sc} \) remained in the CF tracheas, it is possible that a CaCC (but probably not TEM16A, as this residual \( I_{sc} \) is not reduced in Tmem16a/B mice) is responsible for the residual \( I_{sc} \). With respect to regulated Cl⁻ secretion, the pattern in the CF mouse was the inverse of the TEM16A mutant mouse, i.e. forskolin-stimulated \( I_{sc} \) was small in WT mice but reduced more than 75% in CF mice, whereas UTP-stimulated Cl⁻ secretion was large and unaffected by CFTR gene targeting. Note that in the adult CF mouse, there is no defect in either the unstimulated \( I_{sc} \) or the residual \( I_{sc} \), suggesting that CFTR function wanes with age (13).

Interestingly, in mice in which either the cAMP-mediated Cl⁻ channel (Cfr gene) or the calcium-mediated Cl⁻ channel (Tmem16a gene) was deleted, a similar congenital defect in the tracheal cartilage was observed (5, 8, 19). In mice with either mutation, this defect involves the cartilaginous rings on the ventral surface of the trachea. In the Tmem16a/B pups, the cartilaginous rings were discontinuous along the entire length of the trachea. This phenotype, which extends into the mainstem bronchi, was 100% penetrant in the Tmem16a mutant pups (5). This cartilaginous defect was also present in both the CFTR null mutation (cftr<sup>tm1unc</sup>) and the \( \Delta F \) 508 CFTR mutations (cftr<sup>tm1kth</sup>) at a frequency of occurrence ranging from 86 to 100% (8). However, in the cftr<sup>tm1unc</sup> mutation, the defect was confined to the upper trachea, whereas in the cftr<sup>tm1kth</sup> mice, the defect was present all along the trachea (8). Because the two CF mouse models studied were on different genetic backgrounds, the difference in phenotypic expression of the tracheal malformation may be a strain-dependent phenomenon (8).

Two quite different hypotheses may describe the molecular mechanisms producing the similar morphologic findings in mice with different genetic mutations. First, it is possible that both CFTR and TEM16A expression in mesenchymal structures, e.g. tracheal smooth muscle, are important for cartilage development. Both CFTR (20) and TEM16A (5) have been reported to be expressed in tracheal smooth muscle, and we observed expression of TEM16A in the mesenchyme isolated from neonatal tracheal tissue (Fig. 1B). Second, it is possible that the defects reflect a reduction in Cl⁻ (and liquid) secretion into the fetal lung. A reduction in both basal (CFTR) and Ca<sup>2⁺</sup>-regulated (TEM16A) Cl⁻ secretion is predicted to reduce fetal lung liquid secretion and decrease pulmonary transmural pressure gradients. Transmural pressure gradients are important stimuli for lung growth, perhaps including tracheal cartilage.

The presence of mucus (neutral mucopolysaccharide red-stained material in AB-PAS-stained sections) in the lumen of the Tmem16a/B tracheas was striking and not previously reported by Rock et al. (5), possibly due to differences in fixation techniques. A similar accumulation of mucus in the trachea has been observed in a mouse model of ASL depletion caused by transgenic overexpression of βENaC (21). Based on this comparison, we speculate that the mucus accumulation reflects a defect in CaCC-mediated Cl⁻ secretion and consequent ASL depletion.

However, no mucus was observed in the CF tracheas. Although the CF neonatal tracheas exhibit a reduction in the unstimulated secretion by \( \sim 50\% \) and a greater than 75% reduction in the cAMP-stimulated response, the absolute magnitudes of these reductions in Cl⁻ secretion were small (10 and 6 \( \mu A/cm² \), respectively), likely too small to reduce ASL volume and produce a reduction in mucus clearance sufficient to produce mucus accumulation. In contrast, in the Tmem16a/B tracheas, the basal Cl⁻ secretory rate appeared normal, but the reduction in stimulated Cl⁻ secretion via CaCC (TEM16A) was large (\( \sim 40 \mu A/cm² \)). We speculate that it is the large, regulated component of Cl⁻ secretion that is necessary to maintain ASL hydration sufficient for adequate MCC in murine airways.

The identification of both cartilaginous defects and intraluminal mucus accumulation have led us to re-evaluate the mode of early death of Tmem16a/B mice (5). The cartilaginous defects were extensive in the Tmem16a/B tracheas and produced increased compliance that resulted in collapse of Tmem16a/B tracheas with the slightest pressure. Morphometric indications of this “floppiness” were noted in the fixed tracheas of Tmem16a/B mice (Fig. 2B). We speculate that pressure-induced collapse of tracheas caused intraluminal mucus-mediated adhesion of opposing tracheal walls to occur, resulting in acute asphyxia in most neonates (90% of the pups die by day 9 (5)). The reported presence of air in the esophagus and stomach (5) likely is a result of gasping secondary to asphyxia.

In conclusion, we have shown that Tmem16a encodes for the dominant CaCC activity in the superficial epithelium of neonatal murine airways. Based on the accumulation of mucus in airways likely due to deficient Cl⁻ and liquid secretion, and perhaps the relative magnitude of the cartilaginous defects in Tmem16a/B tracheas, TEM16A, not CFTR, appears to be the dominant Cl⁻ channel in mouse airways. We speculate that maneuvers to extend the life of Tmem16a/B mice and/or generation of airway-specific Tmem16a conditional null mice will produce a mouse lung model of CF-like defective Cl⁻ and liquid secretion, intrapulmonary mucus stasis, inflammation, and death.

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