Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel currents in mammary secretory cells from lactating mouse

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MAMMARY GLAND IS AN EXOCRINE GLAND that is physiologically active only in postpartum female mammals. During the lactation period, mammary secretory (MS) cells produce milk containing organic components and ions. Lactose, one of the organic components, is a major osmolyte in milk, and contributes to the determination of milk volume by influencing water secretion (43, 45). Meanwhile, because the ions substantially contribute to the osmolality of milk, it is also believed that ion transport through the transcellular pathway involves the secretion of ionic fluid and thus the regulation of amount and composition of milk (41). Revealing the ion transport system in the MS cells is important for understanding the mechanisms of lactation.

As seen in other exocrine glands like the salivary gland and pancreatic exocrine gland (15, 22, 27), it has been considered that the transepithelial Cl\textsuperscript{−} secretion pathway, which is composed of basolateral transporters for Cl\textsuperscript{−} uptake and apical Cl\textsuperscript{−} channels for excretion, might be present for ionic fluid secretion in the mammary gland (41). Some findings have provided indirect evidence to support this notion. The molecular expression and/or function of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase at the basolateral membrane of acinar cells has been shown in the lactating mammary gland (41). Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter activity has been also demonstrated in mammary tissue explants (40). In addition, Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter 1 protein has been detected on the basolateral membrane of mammary acini of lactating mice, albeit at a lower level than ductal cells in virgin mice (42). These transporters may accumulate Cl\textsuperscript{−} inside the acinar cells, so that the calculated equilibrium potential of Cl\textsuperscript{−} across the apical membrane is more positive than their membrane potentials (24, 41). Although an agonist-stimulated secretion of the ionic fluid of milk has not been proven in vivo, in the experiment using a cultured mouse mammary epithelial cell line, addition of ATP to the basolateral and apical side of the cells augmented the transepithelial potential (apical side negative) and fluid secretion concomitant with the elevation of intracellular Ca\textsuperscript{2+} concentration (5). These changes were inhibited by the apical treatment of the Cl\textsuperscript{−} channel inhibitor, 4,4′-disothiocyanostilbene-2,2′-disulfonic acid (DIDS) (5), suggesting that the Cl\textsuperscript{−} secretion via apical Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel (CaCC) is important for ion secretion and the subsequent osmotic water secretion under the situation where the intracellular Ca\textsuperscript{2+} is elevated. Therefore, apical Cl\textsuperscript{−} channels, especially CaCCs, might be key molecules in the mechanism of Cl\textsuperscript{−} and ionic fluid secretion in lactating mammary epithelia.

The current of the CaCC was first described in the 1980s in Xenopus oocytes and inner segments of salamander photoreceptor (2, 3, 28). Similar channels are expressed in various mammalian epithelial cells including airway, intestinal, salivary gland, and pancreatic epithelia, and contribute to the fluid secretion (13, 27). The CaCC, also called a “classical” CaCC, was characterized by its distinctive properties: the activation with intracellular Ca\textsuperscript{2+} at submicromolar range, the time-dependent activation and deactivation at positive and negative membrane potentials, respectively, the outward rectification, the permeability to other monovalent anions, and the sensitivities to Cl\textsuperscript{−} channel blockers (niflumic acid (NFA), DIDS, etc.) (13). Recent studies identified TMEM16A, which is encoded by Ano1 gene, as the molecular basis of the classical CaCC (6, 39, 48). Heterologously expressed TMEM16A displayed electrophysiological characteristics that were similar to those of the CaCCs in native mammary secretory (MS) cells of lactating animals. We therefore assessed membrane current in MS cells that were freshly isolated from lactating mice using whole cell patch-clamp techniques. In MS cells, we detected CaCC current that exhibited the following characteristics: 1) Ca\textsuperscript{2+}-dependent activation at the concentrations of submicromolar range; 2) voltage-dependent activation; 3) slow kinetics for activation and deactivation; 4) outward rectification of the steady-state current; 5) anion permeability in the sequence of I\textsuperscript− > NO\textsubscript3\textsuperscript− > Br\textsuperscript− > Cl\textsuperscript− >> glutamate; 6) inhibition by Cl\textsuperscript{−} channel blockers (niflumic acid, DIDS, and CaCCinh-A01). These characteristics of native CaCC current were similar to reported characteristics of heterologously expressed TMEM16A. RT-PCR analyses showed the expression of multiple CaCC channels including TMEM16A, Best1, and Best3 in the mammary glands of lactating mice. Immunohistochemical staining revealed the localization of TMEM16A protein at the apical membrane of the MS cells. Collectively, our data strongly suggest that MS cells functionally express CaCC, which is at least partly constituted by TMEM16A. The CaCC such as TMEM16A at the apical membrane of the MS cells may influence the quantity and/or quality of milk.

CaCC; lactation; mammary gland; mammary secretory cell; patch-clamp

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classical CaCC (14, 35). In salivary and pancreatic exocrine glands, TMEM16A expresses at the apical membrane of the acinar cells (16, 48). In vivo knockdown and knockout of TMEM16A in mouse salivary gland have clearly demonstrated that TMEM16A contributes to the CaCC currents and the Ca\(^{2+}\)-mobilizing agonist-induced salivation (7, 38, 48). Thus, it is now known that TMEM16A is a key molecule for the Ca\(^{2+}\)-dependent Cl\(^-\) and subsequent water secretion in the exocrine glands (15, 35). The expression of Ano1 mRNA has been detected in mouse mammary epithelial cells at the late stage of pregnancy (39). However, the functional expression of CaCC in native MS cells of lactating animals remains unclear.

We hypothesized that CaCCs like TMEM16A are active at the apical membrane of MS cells of lactating animals. To test this hypothesis, in this study, we measured a whole cell Cl\(^-\) current that was activated by the physiological concentration of internal Ca\(^{2+}\) in the freshly isolated MS cells of lactating mice and analyzed its properties to explore the molecular basis of the CaCC current in the MS cells. Furthermore, we analyzed the expression of CaCCs using RT-PCR and immunohistochemistry. Our data strongly suggest that the classical CaCC is active in the MS cells and that TMEM16A at the apical membrane of the MS cells may partly contribute to the CaCC current.\(^1\)

**MATERIALS AND METHODS**

**Animals.** Female and male C57BL/6J mice obtained from Nihon SLC (Shizuoka, Japan) and their offspring were used for the experiments. The animal experimental procedures were carried out in accordance with Regulations on Management and Operation of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (OUAVM), and were approved by OUAVM Animal Care and Use Committee. The mice were housed at 23 ± 2°C with a 12:12-h light-dark cycle, and given food and water ad libitum.

**Patch-clamp experiments.** The MS cells were isolated as detailed in our recent work (20). In brief, female mice were killed by cervical dislocation at mid-lactation (day 15.3 ± 0.2 of lactation, n = 57) and their abdominal, inguinal, and/or thoracic mammary glands were collected. The minced mammary glands were incubated in a digestion buffer, which is a divalent cation-free standard bath solution containing (in mM) 145 NaCl, 5 KCl, 10 HEPES, 10 glucose, and 4.6 NaOH at pH 7.4, supplemented with collagenase (type 1, 300 U/ml; Wako, Osaka, Japan) and hyaluronidase (100 U/ml; Sigma-Aldrich, St. Louis, MO), for 30 min at 37°C in a shaking water bath. After gentle trituration with a pipette, the tissue was incubated once more for 30 min in the fresh digestion buffer. The digested tissue was filtered through 100-μm nylon mesh and washed three times with the divalent cation-free standard bath solution.

The isolated MS cells were plated out onto a coverslip in a chamber mounted on an inverted microscope and perfused with a standard bath solution of the following composition (in mM): 145 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1 CaCl\(_2\), 1 MgCl\(_2\), and 4.6 NaOH at pH 7.4. Current recordings were performed with an EPC7 Plus amplifier (HEKA Electronic, Lambrecht, Germany) in the whole cell configuration. The reference electrode was an Ag-AgCl electrode, which was connected to the bath via an agar bridge filled with the standard bath solution. Patch-clamp pipettes were pulled from glass capillaries (G-1.5; Narishige, Tokyo, Japan) using a vertical puller (model PP-830; Narishige) so as to have resistances of 10–20 MΩ. Bath solution changes were accomplished by gravity feed from reservoirs. The results are reported as means ± SE (standard error of the mean) of independent experiments (n), where n refers to the number of cells patched.

**Data analysis.** In the experiments where activation kinetics were analyzed, current traces from 10 to 390 ms of the 400-ms step pulses were fitted to the single exponential function of time (t) plus a constant term equation: I(t) = A exp(−t/τ) + C, where A and C are constant terms and \( \tau \) is the time constant of activation. When the Cl\(^-\) conductance (g\(_{\text{Cl}}\)) of the tail current was determined, the voltage drops of prepulse potentials and tail potentials due to the series resistance were mathematically collected. The conductance was determined using the following equation: g\(_{\text{Cl}}\) = I\(_{\text{tail}}\)/E\(_{\text{Cl}}\) − V\(_{\text{tail}}\), where I\(_{\text{tail}}\), E\(_{\text{Cl}}\), and V\(_{\text{tail}}\) represent the tail current density, the equilibrium potential of Cl\(^-\), and the corrected tail potential, respectively.

In the experiments where the permeability ratio (P\(_{\text{K}}\)/P\(_{\text{Cl}}\)) was estimated, the currents were elicited with the 800-ms ramp pulse [from (V\(_{\text{hold}}\) − 60) mV to (V\(_{\text{hold}}\) + 90) mV, where V\(_{\text{hold}}\) is the holding potential]. Because of the difference of the liquid junction potentials, the values of V\(_{\text{hold}}\) were varied among the bath solutions containing different anions: −45 mV for Cl\(^-\) and −44 mV for Br\(^-\) and NO\(_3^-\), respectively.

\(^1\) This article is the topic of an Editorial Focus by Bruce D. Schultz (39a).
Cl\textsuperscript{−} and −39 mV for glutamate. The permeability ratio (P_{Cl}/P_{NO3}) was calculated from the shift of the reversal potential (ΔV_{rev}) after substitution of most of the extracellular Cl\textsuperscript{−} by foreign anions (Br\textsuperscript{−}, I\textsuperscript{−}, NO\textsubscript{3}\textsuperscript{−}, and glutamate). On the basis of the assumption that the current was only carried by these monovalent anions, ΔV_{rev} was formulated as follows (a derivation from Goldman, Hodgkin, and Katz equation): ΔV_{rev} = RT/F \ln [P_{Cl}^{-1} \cdot (1/[Cl\textsuperscript{−}])_o + P_{NO3} \cdot ([Cl\textsuperscript{−}])_o + P_{X} \cdot ([X\textsuperscript{−}])_o + P_{X}\textsuperscript{3} \cdot ([X\textsuperscript{−}])_o]}. Hence, P_{Cl}/P_{NO3} = ([Cl\textsuperscript{−}])_o/[Cl\textsuperscript{−}]/([Cl\textsuperscript{−}])_o + P_{NO3} ([Cl\textsuperscript{−}])_o/[Cl\textsuperscript{−}]/([Cl\textsuperscript{−}])_o + P_{X} ([X\textsuperscript{−}])_o/[Cl\textsuperscript{−}]/([Cl\textsuperscript{−}])_o + P_{X}\textsuperscript{3} ([X\textsuperscript{−}])_o/[Cl\textsuperscript{−}]/([Cl\textsuperscript{−}])_o + P_{X}\textsuperscript{3}) \cdot ([X\textsuperscript{−}])_o + P_{X}\textsuperscript{3} \cdot ([X\textsuperscript{−}])_o].

Table 1. Primers pairs for CaCC mRNA

<table>
<thead>
<tr>
<th>Target (Gene Name)</th>
<th>Accession No. (Amplified Region)</th>
<th>Primer Pair</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEM16A (Ano1)</td>
<td>NM_178642.5 (471–829)</td>
<td>5’-acctcaacagaggtctcg-3’</td>
<td>359</td>
</tr>
<tr>
<td>TMEM16B (Ano2)</td>
<td>NM_153589.2 (633–881)</td>
<td>5’-gttcctgagttggtc-3’</td>
<td>249</td>
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<tr>
<td>Bestrophin1 (Best1)</td>
<td>NM_011913.2 (1537–1819)</td>
<td>5’-gacctctgtgcctctct-3’</td>
<td>283</td>
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<tr>
<td>Bestrophin2 (Best2)</td>
<td>NM_001130194.1 (84–347)</td>
<td>5’-ctcagcttgagtgatgg-3’</td>
<td>264</td>
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<tr>
<td>Bestrophin3 (Best3)</td>
<td>NM_001007583.1 (2046–2693)</td>
<td>5’-caagttctcagctctct-3’</td>
<td>684</td>
</tr>
<tr>
<td>β-Actin (Actb)</td>
<td>NM_007393.3 (28–246)</td>
<td>5’-aggctacctttgcctct-3’</td>
<td>219</td>
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Cl\textsuperscript{−} and −39 mV for glutamate. The permeability ratio (P_{Cl}/P_{NO3}) was calculated from the shift of the reversal potential (ΔV_{rev}) after substitution of most of the extracellular Cl\textsuperscript{−} by foreign anions (Br\textsuperscript{−}, I\textsuperscript{−}, NO\textsubscript{3}\textsuperscript{−}, and glutamate). On the basis of the assumption that the current was only carried by these monovalent anions, ΔV_{rev} was formulated as follows (a derivation from Goldman, Hodgkin, and Katz equation): ΔV_{rev} = RT/F \ln [P_{Cl}^{-1} \cdot (1/[Cl\textsuperscript{−}])_o + P_{NO3} \cdot ([Cl\textsuperscript{−}])_o + P_{X} \cdot ([X\textsuperscript{−}])_o + P_{X}\textsuperscript{3} \cdot ([X\textsuperscript{−}])_o]. Hence, P_{Cl}/P_{NO3} = ([Cl\textsuperscript{−}])_o/[Cl\textsuperscript{−}]/([Cl\textsuperscript{−}])_o + P_{NO3} ([Cl\textsuperscript{−}])_o/[Cl\textsuperscript{−}]/([Cl\textsuperscript{−}])_o + P_{X} ([X\textsuperscript{−}])_o/[Cl\textsuperscript{−}]/([Cl\textsuperscript{−}])_o + P_{X}\textsuperscript{3} \cdot ([X\textsuperscript{−}])_o/[Cl\textsuperscript{−}]/([Cl\textsuperscript{−}])_o + P_{X}\textsuperscript{3} \cdot ([X\textsuperscript{−}])_o].

Table 2. Primers pairs for the analysis of Ano1 variants

<table>
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<th>Related Segment</th>
<th>Accession No. (Amplified Region)</th>
<th>Sense Primer (Primer Name)</th>
<th>Antisense Primer (Primer Name)</th>
<th>Predicted Product Size, bp</th>
</tr>
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<tbody>
<tr>
<td>Seg. a</td>
<td>NM_178642.5*1 (243–399)</td>
<td>5’-ttggaagagagacagg-3’</td>
<td>(S1)</td>
<td>157</td>
</tr>
<tr>
<td>Seg. b</td>
<td>NM_178642.5 (1032–1262)</td>
<td>5’-ttggaagagagagag-3’</td>
<td>(A1)</td>
<td>231 (b skipping)</td>
</tr>
<tr>
<td>Seg. c and d</td>
<td>NM_178642.5 (1595–1790)</td>
<td>5’-cttcctggagagagag-3’</td>
<td>(S6)*2</td>
<td>297 (b inclusion)</td>
</tr>
<tr>
<td>Seg. c and d</td>
<td>NM_178642.5 (1601–1790)</td>
<td>5’-cttcctggagagagag-3’</td>
<td>(S5)*3</td>
<td>184 (c and d inclusion)</td>
</tr>
<tr>
<td>Seg. b, c, and d</td>
<td>NM_178642.5 (4*–1790)</td>
<td>5’-cttcctggagagagag-3’</td>
<td>(S4)</td>
<td>262 (c skip, d inclusion)</td>
</tr>
<tr>
<td>Seg. b, c, and d</td>
<td>NM_178642.5 (1032–*4)</td>
<td>5’-cttcctggagagagag-3’</td>
<td>(S2)</td>
<td>764 (b and c inclusion, d skipping)</td>
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<tr>
<td>Seg. b, c, and d</td>
<td>NM_178642.5 (1053–1710)</td>
<td>5’-ccagagagagagagagag-3’</td>
<td>(S3)</td>
<td>842 (b, c, and d inclusion)</td>
</tr>
<tr>
<td>Seg. b, c, and d</td>
<td>NM_178642.5 (1053–1710)</td>
<td>5’-gcttgggagagagagag-3’</td>
<td>(A4)</td>
<td>711 (b skipping and c, d inclusion)</td>
</tr>
</tbody>
</table>

\*Ano1 sequence of NM_178642.5 is a variant including exons coding segment a and c, but not b and d.\*2S6 primer does not detect variants including the exon coding segment c.\*3S5 primer does not detect variants skipping the exon coding segment c.\*4S4 and A3 primers bind to the exon coding segment b and d, respectively. Thus there is no binding site in the sequence of NM_178642.5.
RESULTS

Ca2+-activated Cl− current in MS cells. To clarify the functional expression of CaCC in MS cells, we analyzed the whole cell currents of MS cells that were freshly isolated from lactating mouse mammary glands. In whole cell patch-clamp experiments, the MS cells were perfused with the bath solution richly containing NMDG-Cl and with the pipette solution containing NMDG-glutamate and NMDG-CI with various concentrations (0–1 μM) of free Ca2+ ([Ca2+]pipette). The bath and the pipette solutions contained Cl− at concentrations of 154 and 31 mM, respectively. K+ and Na+ in the perfusate were substituted by an organic monovalent cation, NMDG, to reduce cation currents and to clearly detect a Cl− current, because we have shown that MS cells exhibit an inwardly rectifying potassium channel (Kir) 2.1-like potassium current and a cation current that has not yet been fully characterized (20). The currents were elicited by stepping to various test pulses ranging from −105 to +95 mV with 20-mV intervals from the holding potential of −45 mV, followed by the hyperpolarized tail voltage (−105 mV) (Fig. 1A). When the MS cells were perfused with the nominally Ca2+-free pipette solution, the cells showed only small currents (Fig. 1B). At 0.1 μM [Ca2+]pipette, which lies in the range of basal intracellular Ca2+ level (10, 46), ~67% (4 out of 6 cells tested) of the MS cells exhibited time-dependently activated outward currents at positive test potentials more than +35 mV, but little inward current at negative test potentials, as shown in Fig. 1C. The traces of the activated currents were fitted with a single exponential function with time constants of 399 ± 82, 370 ± 106, 346 ± 82, and 445 ± 126 ms at +35, +55, +75, and +95 mV, respectively (n = 4 each). When the cells were perfused with the pipette solution containing 1 μM free Ca2+, the currents were further activated. Most cells showed time-dependent activation and deactivation at depolarized and hyperpolarized test potentials, respectively (Fig. 1D). At 1 μM [Ca2+]pipette, ~88% (21 out of 24 cells) of the cells exhibited time-dependent activation with time constants of 78 ± 16, 78 ± 7, 75 ± 6, and 73 ± 5 ms at +35, +55, +75, and +95 mV, respectively. The membrane time constant arising from series resistance and capacitance (<1 ms) was much smaller than the activation τ. Because the other cells (3 out of 24 cells) showed promptly activated outward currents, their traces could not be fitted with a single exponential function. Thus, the activation kinetics at 1 μM [Ca2+]pipette were faster than at 0.1 μM [Ca2+]pipette and those were not affected by the membrane potentials. The deactivated traces at −105 mV of 19 cells out of 24 cells at 1 μM [Ca2+]pipette were successfully fitted with a single exponential function with a time constant of 58.4 ± 3.8 ms. Due to the time-dependent changes, the steady-state current showed stronger outward rectification compared with the instantaneous current (Fig. 1F).

Figure 1G summarizes the relationships between averaged steady-state currents and test potentials at various [Ca2+]pipette. The inset of Fig. 1G displays a Ca2+ (1 μM)-dependent current that was determined by subtracting the averaged current at nominally zero [Ca2+]pipette from the averaged current at 1 μM [Ca2+]pipette. The elevating concentrations of the intracellular Ca2+ in the submicromolar range activated the outwardly rectifying currents (Fig. 1G). The reversal potentials (Vrev) of the instantaneous and steady-state current at 1 μM [Ca2+]pipette (approximately −39 mV; Fig. 1F) and the Ca2+ (1 μM)-dependent current (approximately −37 mV; Fig. 1G, inset) were close to the Ec50 (−41 mV). Furthermore, the replacement of external Cl− by glutamate decreased the current conduction and abolished the time-dependent outward current (Fig. 1E). Therefore, the Ca2+-activated currents were carried by Cl−. Our data demonstrated that the MS cells exhibit the Ca2+-activated Cl− current (I_{Cl-Ca}) and suggested that the cells functionally express the CaCC.

We further examined the voltage and Ca2+ dependency of the I_{Cl-Ca} by analyzing the tail currents that were elicited by the tail potentials of −105 mV after the test pulses (prepulses) ranging from −105 mV to +95 mV (Fig. 1A). As shown in Fig. 1D, the cells activated by 1 μM [Ca2+]pipette showed remarkable inward tail currents, which were transiently evoked and were gradually decreased due to the time-dependent deactivation of the CaCC. The instantaneous amplitude of the tail current represents the channel activity at the prepulse potential. Plots of the tail-current amplitudes at different [Ca2+]pipette versus the membrane potentials of the prepulses are shown in Fig. 1H. The cells perfused with nominally zero [Ca2+]pipette displayed small tail currents (approximately −2.5 pA/pF) after the hyperpolarized prepulses, which might be associated with the Ca2+- and voltage-independent current including unidentified background currents (Fig. 1H). At the [Ca2+]pipette of 0.1 μM and more, the tail currents were increased by the positive prepulses (Fig. 1H), suggesting that the CaCC in the MS cell is activated in a voltage-dependent manner. We next assessed the effect of internal free Ca2+ at different prepulse potentials (Fig. 1I). The elevating concentration of [Ca2+]pipette increased the tail currents at every prepulse potentials, indicating the Ca2+-dependent activation of CaCC in MS cells. The Ca2+-dependent activation at the positive membrane potentials was greater than that at the negative potentials (Fig. 1I). This might suggest the interaction between Ca2+-dependent and voltage-dependent gating mechanisms. The findings obtained in electrophysiological analyses should be interpreted with caution because our experiments where the series resistance was not electrically...
Fig. 1. Ca\(^{2+}\)-activated Cl\(^{-}\) current in mammary secretory (MS) cells. A–E: representative traces of whole cell currents of MS cells. Currents were elicited by 400-ms voltage steps from \(-105\) mV to \(+95\) mV with 20-mV intervals followed by 400-ms tail potentials of \(-105\) mV (A). Cells were perfused with pipette solution containing different free [Ca\(^{2+}\)] of nominally 0 (B), 0.1 (C), or 1 \(\mu\)M (D and E) and with bath solution richly containing NMDG-Cl (B–D) or the NMDG-glutamate (E). The data of D and E were obtained from the same whole cell configuration. F: relationships between current density and membrane potential of instantaneous and steady-state currents at 1 \(\mu\)M [Ca\(^{2+}\)]_pipette. The current density of instantaneous and steady-state currents was measured at 10 and 380 ms of the test pulse (white arrowhead and arrow in A), respectively. In the inset of F, current densities were normalized by the instantaneous current density at \(+95\) mV in each cell, and the normalized currents were averaged (n = 24). G: relationships between steady-state current densities and membrane potentials of MS cells at different [Ca\(^{2+}\)]_pipette. Whole cell currents at 0, 0.1, 0.3, 0.6, and 1 \(\mu\)M [Ca\(^{2+}\)]_pipette were elicited by the voltage steps (A). The steady-state current was measured at 380 ms of the test pulse (arrow in A) and normalized by the cell capacitance. Relationship between 1 \(\mu\)M [Ca\(^{2+}\)]_pipette-dependent current, which was determined by subtracting the current at 0 \(\mu\)M [Ca\(^{2+}\)]_pipette from that at 1 \(\mu\)M [Ca\(^{2+}\)]_pipette, and membrane potentials is shown (G, inset). H: relationships between instantaneous tail current density and membrane potential of prepulses at different [Ca\(^{2+}\)]_pipette. The tail current was measured at 10 ms after the onset of the tail potential (arrowhead in A) and normalized by the cell capacitance. I: relationship between tail current density after various prepulse potentials and intracellular Ca\(^{2+}\) concentration. J: relationships between the normalized Cl\(^{-}\) conductances (g\(_{Cl}\)) of the tail currents and the prepulse potentials in various [Ca\(^{2+}\)]_pipette are summarized. The voltage drop of prepulse potentials and tail potentials due to the series resistance was mathematically collected. Each point represents the mean ± SE (n = 6, 6, 12, 10, and 24 for 0, 0.1, 0.3, 0.6, and 1 \(\mu\)M [Ca\(^{2+}\)]_pipette, respectively) in F–J. Error bars of some data points are hidden behind the graph symbol.
compensated might have an error due to a voltage drop. To correct the error, we determined the relationship between the membrane potentials of prepulses and tail-current conductances using the mathematically corrected actual membrane potentials (Fig. 1A). The corrected relationship also indicated the apparent Ca²⁺- and voltage-dependent activation of the \( I_{\text{Cl-Ca}} \) in the MS cells (Fig. 1J).

In our experiments, whole cell currents activated with [Ca²⁺]pipette more than 1 μM could not be recorded with confidence because of the instability of the currents possibly due to the resealing of a patched membrane. Therefore, we could not estimate the maximally activated \( I_{\text{Cl-Ca}} \), the dissociation constant \( K_\varepsilon \) for Ca²⁺, the voltage of half-maximal activation \( V_{1/2} \), and the relationship between the Ca²⁺ sensitivity and the voltage sensitivity in a quantitative manner.

To further characterize the \( I_{\text{Cl-Ca}} \) in the MS cells, the permeability sequence of monovalent anions was determined by replacing external Cl⁻ with I⁻, NO₃⁻, Br⁻, or glutamate and by measuring a \( V_{\text{rev}} \) shift in separate cells (Fig. 2, A–D). For this experiment, the \( I_{\text{Cl-Ca}} \) was activated with the pipette solution containing 31 mM Cl⁻ and 1 μM Ca²⁺ and was elicited by the ramp pulse protocol. In the bath solution containing 154 mM Cl⁻ and the solution containing 150 mM I⁻, NO₃⁻, or Br⁻ instead of 150 mM Cl⁻, the MS cells showed the currents with slight outward rectifications (Fig. 2, A–C). In the bath solution containing 150 mM glutamate, the MS cells showed a linear \( I-V \) relationship with small conductances (Fig. 2D). The replacement of external Cl⁻ with I⁻, NO₃⁻, Br⁻ and glutamate induced the shifts of \( V_{\text{rev}} \) by \(-16.3 \pm 2.4 \) (n = 6), \(-14.0 \pm 2.9 \) (n = 6), \(-8.4 \pm 1.0 \) (n = 7), and \(+36.3 \pm 6.6 \) (n = 7) mV, respectively, from the \( V_{\text{rev}} \) in the solution with 154 mM Cl⁻ (\(-35.8 \pm 1.8 \) mV, n = 26). Using the Goldman, Hodgkin, and Katz equation, the relative permeabilities of these monovalent anions \( (P_x/P_{\text{Cl}}) \) were estimated to be \( 1.94 \pm 0.19, 1.79 \pm 0.19, 1.40 \pm 0.05 \) and \( 0.28 \pm 0.09 \) for I⁻, NO₃⁻, Br⁻, and glutamate, respectively (Fig. 2E). Furthermore, we compared the \( V_{\text{rev}} \) between the bath solution containing 150 mM NMDG-I and 150 mM NMDG-NO₃ in a single cell to confirm the difference of the permeabilities between I⁻ and NO₃⁻. The \( V_{\text{rev}} \) in the NMDG-I solution was more negative than that in the NMDG-NO₃ solution in all the cells that we tested (\(-3.2 \pm 1.3 \) mV, \( n = 4 \)). These results revealed that the permeability sequence of the \( I_{\text{Cl-Ca}} \) in the MS cells was I⁻ > NO₃⁻ > Br⁻ > Cl⁻ >> glutamate.

Collectively, our findings suggest that the native MS cells functionally express CaCC, which shows the Ca²⁺-dependent activation at submicromolar concentrations, the voltage-dependent activation, the outward rectification of the steady-state \( I-V \) relationship, the slow kinetics for activation and deactivation, and the following permeability sequence to anions: I⁻ > NO₃⁻ > Br⁻ > Cl⁻ >> glutamate. These characteristics of the CaCC in MS cells were similar to the classical CaCC recorded in various epithelial cells (13) and heterologously expressed TMEM16A (14, 35).

Fig. 2. Anion selectivity of Ca²⁺-activated Cl⁻ current \( (I_{\text{Cl-Ca}}) \) in MS cells. A–D: effects of anion substitution on \( I_{\text{Cl-Ca}} \) in MS cells. MS cells were perfused with pipette solution containing 1 μM [Ca²⁺] and bath solution richly containing NMDG-Cl. Cl⁻ in the bath solution was replaced by I⁻ (A), NO₃⁻ (B), Br⁻ (C), or glutamate (D). The currents were elicited by 800-ms ramp pulses from −105 (−104 and −99) to +45 (+46 and +51) mV from a holding potential of −45 (−44 and −39) mV in the NMDG-Cl and −1 (−Br and −NO₃), and −glutamate, respectively) bath solution. The current was normalized by that in the NMDG-Cl bath solution at +40 mV in each cell. The averaged normalized currents are shown. Insets show representative current-voltage \( (I-V) \) relationship before and after anion replacement. E: permeability ratio to anions. Relative permeability of these monovalent anions \( (P_x/P_{\text{Cl}}) \) was estimated using the Goldman, Hodgkin, and Katz equation (see METHODS AND MATERIALS). Data are means ± SE (n = 6, 6, 7, and 6 for I⁻, NO₃⁻, Br⁻, and glutamate, respectively).
Pharmacological properties of Ca$^{2+}$-activated Cl$^{-}$ current in MS cells. We next investigated the pharmacological properties of the I$_{Cl, Ca}$ in the MS cells. In this experiment, broad-spectrum classical Cl$^{-}$ channel blockers including NFA and DIDS and a newly found CaCC-selective blocker, CaCCinh-A01 (29), were utilized. The currents were activated by 0.6 μM [Ca$^{2+}$]pipette and recorded with the ramp pulse protocol (from −105 to +95 mV) at pre- and post-treatment of blockers (insets of Fig. 3, A, B, and D). The relationships between the averaged normalized current (I$_{norm}$) and membrane potentials were summarized (Fig. 3, A, B, and D). NFA at 100 μM effectively inhibited the I$_{Cl, Ca}$ (Fig. 3A). The I$_{norm}$ at +90 mV were 0.93 ± 0.03, 0.79 ± 0.05, 0.40 ± 0.03, 0.18 ± 0.02, and 0.17 ± 0.07 with NFA at concentrations of 0.01, 1, 10, 100, and 300 μM, respectively, and the K$_d$ value of NFA for the I$_{Cl, Ca}$ at +90 mV was estimated to be 3.7 μM (Fig. 3C). DIDS inhibited the outward I$_{Cl, Ca}$ at a concentration of 3 mM (Fig. 3B). With DIDS at 10, 100, 300, 1,000, and 3,000 μM, the I$_{norm}$ at +90 mV were 0.95 ± 0.02, 0.92 ± 0.01, 0.85 ± 0.09, 0.46 ± 0.08, and 0.21 ± 0.02, respectively, and the K$_d$ value of DIDS was estimated to be 805 μM (Fig. 3C). The treatment of CaCCinh-A01 at a concentration of 100 μM suppressed the I$_{Cl, Ca}$ (70.3 ± 1.1% inhibition at +90 mV) (Fig. 3D). The effects of the blockers, especially CaCCinh-A01, were partially irreversible (Fig. 3, A, B, and D). The inhibitory effect of NFA and DIDS, but not CaCCinh-A01, showed a voltage dependency (Fig. 3E).

Fig. 3. Effects of Cl$^{-}$ channel blockers on I$_{Cl, Ca}$ in MS cells. A and B: effects of niflumic acid (NFA) (A) and DIDS (B) on I$_{Cl, Ca}$ in MS cells. Whole cell currents were recorded with 1,000-nm ramp potentials from −105 to +95 mV with the 0.6 μM Ca$^{2+}$ pipette solution in the NMDG-Cl bath solution (Control), the solution with 100 μM NFA or 3 mM DIDS, and the fresh NMDG-Cl solution (Wash). The currents were normalized by the Control current at +90 mV in the same cell. Each point represents the mean ± SE (n = 3–6). The error bars of some data points are hidden behind the graph symbol. Representative I–V relationships of the Control and inhibited currents are shown in the insets. C: the dose dependency of NFA and DIDS inhibition. The normalized currents at +90 mV with various concentrations of NFA (filled circles) or DIDS (filled triangles) were plotted. Each point represents the mean ± SE (n = 3–6). The plots were fitted to the Hill equation: $I_{norm} = I_{min} + (1 - I_{min})/[1 + ([Inh]/K_d)^h]$, $I_{min} = 0.14$, h = 0.8, and K$_d = 2.7 \times 10^{-5}$ M for NFA, $I_{min} = 0.09$, h = 1.5, and K$_d = 805 \times 10^{-6}$ M for DIDS. D: effect of CaCCinh-A01 on I$_{Cl, Ca}$ in MS cells. Whole cell currents were recorded in the NMDG-Cl solution with or without CaCCinh-A01 (100 μM, n = 4) and averaged normalized currents are shown. Data represent means ± SE. Inset shows representative I–V relationships. E: voltage dependency of inhibitors. The fractions of inhibited current after treatment of NFA (100 μM), DIDS (3 mM), and CaCCinh-A01 (100 μM) at −90 and +90 mV are shown. Data represent means ± SE (n = 4–5). *P < 0.05 vs. −90 mV.

mRNA expression of CaCC in lactating mammary glands. The electrophysiological and pharmacological analyses suggested the functional expression of CaCC, particularly TMEM16A, in the MS cells. Thus expression of the Ano1 gene, which encodes TMEM16A, and other CaCC genes (Ano2, Best1, Best2, and Best3) was tested. RT-PCR analyses showed abundant expression of Ano1 mRNA in the mammary glands of lactating mice (Fig. 4B). Additionally, Best1 and Best3, but not Ano2 and Best2, mRNAs were detected in lactating mammary glands (Fig. 4B).

It has been reported that human TMEM16A has multiple functional isoforms generated by the combination of alternative promoters and alternative three exons (35). The variant that lacks the first ATG (start codon; at 306–308 in the hAN01 sequence of accession no. NM_018043.5) is translated into a protein lacking the N-terminal amino acid sequence labeled segment a, and variants that include or skip the three alternative exons translated into the variants with or without segment b, c, and d. Homologous variants were also reported in mouse tissues (8, 17, 32). We therefore examined the mouse Ano1 (mAno1) variants that are expressed in lactating mammary glands with specific primer pairs (Table 2 and Fig. 4A). First, the presence of mAno1 mRNA including the first ATG was assessed. We detected the 5’-region harboring the start codon (ATG at 269–271 in the mAno1 sequence of accession no. NM_178642.5) that corresponds to the first ATG in human

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**Figure 3.** Effects of Cl$^{-}$ channel blockers on I$_{Cl, Ca}$ in MS cells. A and B: effects of niflumic acid (NFA) (A) and DIDS (B) on I$_{Cl, Ca}$ in MS cells. Whole cell currents were recorded with 1,000-nm ramp potentials from −105 to +95 mV with the 0.6 μM Ca$^{2+}$ pipette solution in the NMDG-Cl bath solution (Control), the solution with 100 μM NFA or 3 mM DIDS, and the fresh NMDG-Cl solution (Wash). The currents were normalized by the Control current at +90 mV in the same cell. Each point represents the mean ± SE (n = 3–6). The error bars of some data points are hidden behind the graph symbol. Representative I–V relationships of the Control and inhibited currents are shown in the insets. C: the dose dependency of NFA and DIDS inhibition. The normalized currents at +90 mV with various concentrations of NFA (filled circles) or DIDS (filled triangles) were plotted. Each point represents the mean ± SE (n = 3–6). The plots were fitted to the Hill equation: $I_{norm} = I_{min} + (1 - I_{min})/[1 + ([Inh]/K_d)^h]$, $I_{min} = 0.14$, h = 0.8, and K$_d = 2.7 \times 10^{-5}$ M for NFA, $I_{min} = 0.09$, h = 1.5, and K$_d = 805 \times 10^{-6}$ M for DIDS. D: effect of CaCCinh-A01 on I$_{Cl, Ca}$ in MS cells. Whole cell currents were recorded in the NMDG-Cl solution with or without CaCCinh-A01 (100 μM, n = 4) and averaged normalized currents are shown. Data represent means ± SE. Inset shows representative I–V relationships. E: voltage dependency of inhibitors. The fractions of inhibited current after treatment of NFA (100 μM), DIDS (3 mM), and CaCCinh-A01 (100 μM) at −90 and +90 mV are shown. Data represent means ± SE (n = 4–5). *P < 0.05 vs. −90 mV.
ANO1 (hANO1) (Fig. 4C; primers S1 and A1). Next, three alternative spliced variants were examined. Primer pair spanning the exon encoding the segment b (exon b) amplified both-inclusion and b-skipping transcripts (Fig. 4C; primers S2 and A2). Semiquantitative comparison by densitometry showed that the expression level of b-inclusion transcripts was approximately 1.3-fold (1.28 ± 0.13, n = 3) greater than that of b-skipping transcripts. The c-skipping variants were detected in brain but not in lactating mammary glands (Fig. 4C; primers S6 and A5). When exon d-spanning primers were used, abundant d-skipping transcripts and lesser d-inclusion transcripts (0.22 ± 0.12 in abundance relative to d-skipping transcripts) were amplified (Fig. 4C; primers S5 and A5). When the primer set of sense primer binding to the exon b (primer S4) and the antisense primer binding to the 3'-flanking region of exon d (primer A5) or the sense primer binding to the 5'-flanking region of exon b (primer S2) and the antisense primer binding to the exon d (primer A3) were utilized, bcΔd, Δbcd, and bcd fragments were amplified (Fig. 4C). Because the bands of the bcd variant were faint, we further confirmed their expression by using primer pairs for the nested PCR, S2 and A3 followed by S2 and A2 (Fig. 4C). Moreover, when the sense and antisense primers binding to b-skipping and d-skipping sequence, respectively, were used, we detected amplification (Fig. 4C; primer S3 and A4). These results showed that multiple Ano1 transcripts encoding TMEM16A isoforms including at least TMEM16A (ac), (abc), (acd), and (abcd) are present in the mammary gland of lactating mice.

Protein expression of TMEM16A in lactating mammary gland. We next examined the localization of TMEM16A in the lactating mammary gland. As shown in Fig. 5A, the mammary glands of lactating mice were mainly occupied by mammary parenchymal tissue such as lobuloalveolar and ductal structures. The secondary and/or tertiary ductal structures were surrounded by thick stroma such as fibroblast and collagen fiber, while the lobuloalveolar tissues were surrounded by thinner stroma (Fig. 5A), as reported previously (37). The cuboidal luminal cells, i.e., MS cells, in both the acinar and ductal structures were enlarged with a large cytoplasm and some of the cells contained milk fat globules (Fig. 5). The sections were immunohistochemically stained with anti-TMEM16A antibody (Fig. 5, A and B) or the control IgG (Fig. 5C). The anti-TMEM16A antibody used in this experiment, which was generated using the human TMEM16A peptide (ranging between aa 100–450) as an immunogen, detects multiple mTMEM16A variants, including ab, ac, and abc variants, at the cell surface (11). A minor fraction of the epithelial cells (0.9 ± 0.2%, n = 3) were most densely stained at the cytosol region and the apical membrane (Fig. 5, A and B; arrowheads). Such densely stained cells were small in size with a narrow cytoplasm and did not include lipid droplets inside (Fig. 5, A and B). Because of the low occurrence and unusual shape, these types of cells were not used for patch-clamp analyses. The nature of the densely stained cells remains to be elucidated. The majority of MS cells in both acinar and ductal structures displayed the positive reaction to anti-TMEM16A.
antibody staining at the apical membrane (Fig. 5, A and B; arrows). These results demonstrate that TMEM16A is expressed at the apical membrane of MS cells in the lactating mammary gland.

**DISCUSSION**

It has been hypothesized that the Cl− channel at the apical membrane of MS cells contributes to the Cl− and subsequent water secretion into the lumen of the mammary gland. However, there is no direct evidence for the functional expression of Cl− channels in the MS cells of lactating mice. The CaCC exhibited the following characteristics: 1) Ca2+-dependent activation; 2) voltage-dependent activation; 3) time-dependent activation and deactivation; 4) outward rectification of the steady-state current; 5) permeability ratios in the sequence of I− > NO3− > Br− > Cl− >> glutamate; and 6) sensitivity to Cl− channel blockers such as NFA, DIDS, and CaCinh-A01.

It has been well known that TMEM16A, one of the CaCC molecules, plays important roles in the Cl− secretion in exocrine glands (15, 35). To assess the possibility that the CaCC current in the MS cells might be conducted by TMEM16A, the electrophysiological and pharmacological characteristics of the MS-cell CaCC current were compared with the reported properties of TMEM16A and the classical CaCC. First, the I-V relationship of the steady-state CaCC current in the MS cells showed an outward rectification due to the slow activation and deactivation at positive and negative membrane potentials, respectively (Fig. 1, D and F), that agreed with the features of TMEM16A (14, 33, 35). However, a few MS cells (3 out of 24 cells at 1 μM [Ca2+]i) showed instantaneous activation and little time-dependent activation. This cell-to-cell variation in the activation kinetics might be due to the different ratio of TMEM16A variants in each of the MS cells, because Ferrera et al. (11) have shown that lack of segment b resulted in larger instantaneous current and smaller time-dependent activation. We actually detected both b-inclusion and -skipping variants in lactating mammary glands (Fig. 4C). Second, tail-current analyses showed that the CaCC in the MS cells was activated by intracellular Ca2+ at submicromolar concentrations (Figs. 1f). Because the activation level at 1 μM [Ca2+]i did not reach the maximum level, the Kd value of the Ca2+-dependent activation would be >300 nM at +95 mV in the MS cells. It has been revealed that the Ca2+ sensitivity of hTMEM16A (ac) (Kd = 85 nM at +100 mV) was nearly fourfold higher than that of the hTMEM16A (abc) variant (Kd = 332 nM at +100 mV) (11). Romanenko et al. (38) have examined the Ca2+ sensitivity of mTMEM16A (ac) and calculated the Kd value as 196 nM at +120 mV. Thus, the Ca2+ sensitivity of the CaCC current in the MS cells may lie within the range of mTMEM16A variants including (ac) and (abc). Lastly, the permeability sequence of the CaCC in MS cells was determined as I− > NO3− > Br− > Cl− >> glutamate (Fig. 2E). These characteristics were consistent with previous reports for the heterologously expressed mTMEM16A (ac) (38), mTMEM16A (0) (31), and hTMEM16A (ab) (19).

The potency of classical Cl− channel inhibitors, NFA and DIDS, for the CaCC current in MS cells was also similar to that for mammalian TMEM16A. Both drugs inhibited the Ic1,cs in the MS cells, and the block by NFA (Kd = 3.7 μM at +90 mV) was more potent than DIDS (Kd = 805 μM at +90 mV) in the MS cells (Fig. 3, A–C). The higher potency of NFA compared with DIDS has also been reported for heterologously expressed TMEM16A. For instance, the Kd values of NFA and DIDS for hTMEM16A (abc) were 7.4 μM and 549 μM, respectively, at +80 mV (25). mTMEM16A (ac) displayed higher sensitivity to NFA (Kd ≈ 30 μM) compared with DIDS (Kd ≈ 300 μM) (38). In our experiment, the value of the Hill coefficient for NFA was estimated to be 0.8, close to unity, likely indicative of pore block. On the other hand, the Hill coefficient for DIDS was estimated to be 1.5. This does not exclude the possibility of the presence of multiple binding sites for DIDS. Thus, there might be different blocking mechanisms between NFA and DIDS for the CaCC in the MS cells. However, the precise mechanism has not been elucidated in this study. Also for heterologously expressed mammalian TMEM16A, the blocking mechanisms of these inhibitors have not been conclusively shown and the value of the Hill coefficient for DIDS has not been available at present.

Collectively, the characteristics of the CaCC current in the MS cells were, at least qualitatively, similar to those of TMEM16A variants (ac, abc, and acd). Thus, our data strongly suggest that TMEM16A partly contributes to the CaCC current in the MS cells. Actually, transcripts of these variants were detected in lactating mammary gland with RT-PCR analyses (Fig. 4C).

Do other CaCCs contribute to the Ic1,cs? It has been known that membrane proteins such as TMEM16B, Best1, Best2, and Best3 are activated with the submicromolar [Ca2+]i, and function as a CaCC (26, 36, 39, 44, 47). Because the mRNA
expression of TMEM16B and Best2 was negligible in the mammary glands of lactating mice, it is likely that these two molecules have little involvement in the \(I_{\text{Cl-Ca}}\) in the MS cells. mRNA expression of Best1 and Best3 was detected in the lactating mammary gland. However, the pharmacological properties of the CaCC in the MS cells were different from those of Best1 and Best3. Best1 has lesser sensitivity to NFA (\(K_d = 102\) µM at +80 mV) and greater sensitivity to DIDS (\(K_d = 3.9\) µM at +80 mV) than TMEM16A (25). Also, the current of a native Best3-like channel in mammalian cells was not inhibited by 100 µM NFA and was pharmacologically distinguishable from NFA (100 µM)-sensitive TMEM16A-like current (26). Thus, it is suggested that the contribution of Best1 and Best3 to the \(I_{\text{Cl-Ca}}\) in the MS cells was small. However, the relatively higher permeability to glutamate (Fig. 2E) than heterologously expressed TMEM16A (34) and residual current after addition of NFA (100 µM) (Fig. 3A) may imply that Best currents were subtly included in the \(I_{\text{Cl-Ca}}\) of the MS cells. Moreover, we cannot exclude the possibility that other unidentified currents such as a current at nominally zero \([\text{Ca}^{2+}]\) affected the analyses of properties of the native CaCC in the MS cells. Studies using mammary gland-specific CaCC (TMEM16A, Best1, or Best3) knockout mice would be helpful to further examine the contribution of these channels to the \(I_{\text{Cl-Ca}}\) in the MS cells.

It is worth discussing the physiological significance of the CaCC in lactating mammary gland. The CaCC in MS cells may contribute to \(\text{Ca}^{2+}\)-activated \(\text{Cl}^-\) secretion and the subsequent osmotic water secretion and may finally modulate the volume and composition of milk. Although such a stimulated secretion of the ionic fluid by the intracellular \(\text{Ca}^{2+}\) recruitment in the MS cells has not been proven in vivo, the present study and previous reports may support the validity of this secretion model. First, as shown in Fig. 1, \(H\) and \(I\), the activity of the CaCC in MS cells was regulated at physiological levels of \([\text{Ca}^{2+}]\) at negative membrane potentials, which are near the reported resting membrane potential of the apical membrane (4, 23). It has been reported that the intracellular concentration of \(\text{Ca}^{2+}\) is elevated by extracellular purine nucleotides, which is released by mechanical stress in autocrine and/or paracrine manner in cultured mammary tumor cells (10). Thus, when the MS cells receive the mechanical stress during the milk ejection process (i.e., acini contraction), the purine nucleotides may elevate intracellular \(\text{Ca}^{2+}\) and then modulate the CaCC activity. However, we should note that the role of the purinergic stimuli for the \([\text{Ca}^{2+}]\) elevation in native MS cells remains controversial (46). Second, TMEM16A, a convincing candidate of the CaCC in MS cells, was located at the apical membrane of MS cells in lactating mammary glands (Fig. 5). Based on the data of the intracellular \([\text{Cl}^-]\) [62 mM in guinea pig (23)] and milk \([\text{Cl}^-]\) [42 mM in mice (18), 12–68 mM in guinea pig (1, 23)], the equilibrium potential of \(\text{Cl}^-\) (\(E_{\text{Cl}}\)) at apical membrane calculated to be from −2 to +44 mV. It is speculated that \(\text{Cl}^-\) has an outward electrochemical driving force at the apical membrane because its membrane potential is reported as −44 mV in guinea pig (23) and −14 mV in mice (4). Thus, \(\text{Cl}^-\) may be secreted to the lumen when the apical TMEM16A is activated. It has been demonstrated that DIDS (0.5 mM)-sensitive CaCC contributes to the purinergically stimulated \(\text{Cl}^-\) secretion in the monolayer culture of a mouse mammary epithelial cell line (4). This study is evidence for the \(\text{Ca}^{2+}\)-stimulated secretion model in mammary epithelial cells. However, the inward current of the native CaCC (i.e., \(\text{Cl}^-\) efflux) was not inhibited by 3 mM DIDS in the MS cells (Fig. 3E). The CaCC shown in our study may be different from the channel reported in cultured mammary epithelial cells. Third, the Kir2.1-like Kir channel (20) and the \(\text{Ca}^{2+}\)-activated K+ channels (9, 12) were detected in native MS cells and primary cultured mammary epithelial cells, respectively. The potassium efflux through such potassium channels may contribute to maintain the driving force for \(\text{Cl}^-\) secretion via the CaCC. Further studies will be necessary to verify the \(\text{Ca}^{2+}\)-stimulated secretion model and its molecular basis in lactating mammary gland.

We should pay attention that the amount and composition of milk may not be accounted for solely by the ionic transport model proposed in other exocrine glands including the salivary and pancreatic gland because the mammary gland has distinctive features. Milk contains substantial carbohydrate including lactose (approximately 60 and 200 mM in mice and human, respectively) as an osmolyte. Thus, the carbohydrate secretion also affects the amount and composition of milk (43, 45). Moreover, because produced milk is stored in acini until it is sucked by pups, the milk composition can be modulated after production by the MS cells during the storage. Further studies will be needed to fully understand the molecular mechanisms where the composition of milk is determined. It has also been known that the ionic composition of milk is varied among mammalian species (e.g., higher \(\text{Cl}^-\) concentration of milk in rodents than humans and ruminants), suggesting the difference of mechanisms for transepithelial ion secretion between species. Thus, investigating the difference of CaCC activity and its role in lactation between species will be of interest.

In conclusion, we have demonstrated the functional expression of CaCC in the MS cells freshly isolated from lactating mice. Our results strongly suggest that TMEM16A at least partly contributes to the CaCC current in MS cells. It would be of interest to investigate the role of TMEM16A-like CaCC in stimulated \(\text{Cl}^-\) and ionic fluid secretion in MS cells during lactation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

A.K. and T.I. conceived and designed the research; A.K., O.I., and J.S. performed experiments; A.K., O.I., and J.S. analyzed data; A.K. interpreted results of experiments; A.K. prepared figures; A.K. drafted manuscript; A.K. and T.I. edited and revised manuscript; A.K., O.I., J.S., and T.I. approved final version of manuscript.

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