Permeabilization via the P2X7 Purinoreceptor Reveals the Presence of a Ca\(^{2+}\)-activated Cl\(^{-}\) Conductance in the Apical Membrane of Murine Tracheal Epithelial Cells

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Calcium-activated Cl\(^{-}\) secretion is an important modulator of regulated ion transport in murine airway epithelium and is mediated by an unidentified Ca\(^{2+}\)-stimulated Cl\(^{-}\) channel. We have transfected immortalized murine tracheal epithelial cells with the cDNA encoding the permeabilizing P2X7 purinoreceptor (P2X7-R) to selectively permeabilize the basolateral membrane and thereby isolate the apical membrane Ca\(^{2+}\)-activated Cl\(^{-}\) current. In P2X7-R-permeabilized cells, we have demonstrated that UTP stimulates a Cl\(^{-}\) current across the apical membrane of CF and normal murine tracheal epithelial cells. The magnitude of the UTP-stimulated current was significantly greater in CF than in normal cells. Ion substitution studies demonstrated that the current exhibited a permselectivity sequence of Cl\(^{-}\) > Br\(^{-}\) > gluconate\(^{-}\). We have also determined a rank order of potency for putative Cl\(^{-}\) channel blockers: niflumic acid ≥ 5-nitro-2-(3-phenylpropylamino)benzoic acid > 4,4’-diisothiocyanostilbene-2,2’-disulfonate > glybenclamide >> diphenylamine-2-carboxylate, tamoxifen, and p-tetra-sulfonato-tetra-methoxy-calix[4]arene. Complete characterization of this current and the corresponding single channel properties could lead to the development of a new therapy to correct the defective airway surface liquid in cystic fibrosis patients.

Chloride secretion across the airway epithelium can be stimulated by a number of secretagogues that activate distinct second messenger transduction mechanisms (reviewed in Refs. 1 and 2). The cystic fibrosis (CF) gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), accounts for the cAMP-regulated apical Cl\(^{-}\) conductance (3, 4). There is, however, compelling evidence that a separate Ca\(^{2+}\)-activated apical Cl\(^{-}\) conductance (CaCC) exists. A large class of ligands, including histamine (5, 6), bradykinin (7, 8), and extracellular ATP (9–11), has been shown to activate CaCC across the apical membrane of airway epithelia. The unique identity of this pathway in airway epithelia was established in studies of CF nasal epithelia, which demonstrated that Ca\(^{2+}\) ionophores are effective Cl\(^{-}\) secretagogues in CF tissues (12–14). Moreover, in the airways of the CFTR(−/−) knockout mouse, which definitively lacks CFTR (15), not only is the CaCC pathway preserved, but it appears to be up-regulated (16).

In the airway epithelium, Cl\(^{-}\) secretion is dependent on the development of a favorable driving force, because at basal conditions Cl\(^{-}\) is at or near electrochemical equilibrium across the apical membrane. Ca\(^{2+}\), can stimulate Cl\(^{-}\) secretion by multiple mechanisms. Elevation of Ca\(^{2+}\), can directly activate an apical membrane-localized Cl\(^{-}\) conductance and thereby stimulate an apical exit pathway for Cl\(^{-}\) secretion. Ca\(^{2+}\)- mobilizing agents can also cause a hyperpolarization in the cell to generate a driving force for Cl\(^{-}\) secretion across the apical membrane by either (or both) inhibiting an apical membrane Na\(^{+}\) conductance (17) or activating a basolateral K\(^{+}\) conductance. Thus study of Ca\(^{2+}\)-activated Cl\(^{-}\) conductance in the apical membrane of a polarized epithelium requires a means to identify the contributions of apical Cl\(^{-}\) conductance in isolation from other actions.

Previous studies in non-polarized secretory epithelium, e.g. airway epithelia or T\(_{84}\) cells plated as isolated or dissociated cells, have shown outwardly rectifying Cl\(^{-}\) currents stimulated by intracellular Ca\(^{2+}\) and sensitive to 4,4’-diisothiocyanostilbene-2,2’-disulfonate (DIDS) (18–24). These descriptions have included such a wide range of Cl\(^{-}\) channel characteristics that no consensus on the characteristics of this channel can be achieved (25–28). Recently, a family of putative CaCC genes has been cloned (29–32). The single channel properties and the cellular localization of these gene products, however, have not yet been determined. Thus, no indisputably apical Ca\(^{2+}\)-activated Cl\(^{-}\) channel has been identified at either the molecular or single channel level.

We have recently identified a CaCC current expressed in immortalized CF and normal murine tracheal epithelial cell lines (33). In the current study we used permeabilization of the basolateral membrane to determine the basic biophysical properties of the CaCC current in a functionally isolated apical membrane of airway epithelial cells. We have accomplished basolateral permeabilization by a novel approach involving stable transfection of the P2X7 purinoreceptor (P2X7-R) into our murine CF tracheal epithelial cell line. The P2X7-R is
unique within its family, because binding of nucleotides (ATP$^+$ is the preferred agonist) to this receptor results in the formation of a membrane pore that is capable of conducting molecules as large as 900 daltons (34, 35). The pore is not ion-selective and allows for free diffusion of both cations and anions. Thus, by application of ATP selectively to the basolateral solution, we can selectively permeabilize this barrier.

We report here the characterization of CaCC in the apical membrane of a CF tracheal epithelial cell line when activated by different classes of Ca$^{2+}$-mobilizing agents (UTP and ionomycin). We have determined the halide selectivity and inhibitor sensitivity of this Cl$^-$ current unambiguously localized to the apical membrane. Importantly, these observations will provide us with the hallmark characteristics for comparison with subsequent whole cell and single channel studies and will enable us to evaluate CaCC candidate genes. A greater understanding of the characteristics and mechanism of regulation of the CaCC pathway is essential for development of pharmacological therapies designed to use CaCC as an alternate Cl$^-$ channel to replace the defective CFTR.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—These studies utilized the immortalized murine tracheal epithelial cell line (MTE18) derived from the CFTR$^{-/-}$ knock-out mouse, described previously (33). Cells were maintained and cultured at 33 °C, the permissive temperature for the immortalizing tsA58 TAg activity (33) on “Transwell-col” culture inserts. Culture medium consisted of a 1:1 mix of Ham’s F12 and 3T3 fibroblast-conditioned medium supplemented with the following hormones: transferrin (2.5 mg/ml), insulin (5 mg/ml), epidermal growth factor (12.5 ng/ml), hydrocortisone (0.5 mg/ml), and CaCl$_2$ (0.5 mM). Cells were harvested for experimental studies by trypsinization and plated at high density (2 × 10$^5$ cells per cm$^2$) on Transwell collagen matrices (Costar Inc.) with a 4.5-mm plating diameter and monitored for confluence by daily monitoring of transepithelial resistance ($R_T$) and potential difference ($V_P$). Only monolayers generating at least a 1.0-millivolt (mV) $V_P$ and a 100-Ωcm$^2$ resistance (after the resistance of the permeable support is subtracted) were used for Ussing chambers studies, typically 5–7 days after plating.

**Ussing Chamber Studies**—Electrical measurements, i.e. $V_P$, $R_T$, and short-circuit current ($I_{sc}$), were made on cell monolayers mounted in Ussing chambers. Monolayers were bathed in a Krebs bicarbonate Ringer solution (KBR) on both the luminal and the serosal sides. Serosal Ca$^{2+}$ was buffered to 300 mM with the addition of EGTA (achieved by the addition of 1 mM EGTA and 0.925 mM Ca$^{2+}$). Other alterations to the bathing solutions are listed in the figure legends. Further details are described in Materials and Methods. All experiments were performed under voltage clamp conditions (clamped to 0 mV) in the presence of a Cl$^-$ gradient followed by the addition of mucosal UTP. Our experimental protocol defines the CaCC current as $I_{CaCC} = I_{UTP} - I_{KBR}$. Because the transepithelial potential is clamped to 0 mV, the equilibrium potential for Cl$^-$ can be calculated as $E_{Cl^-} = -\frac{R_T}{C_{sc}} \log \left(\frac{[Cl^-]}{[Cl^-]}\right)$ and is determined by the chemical driving force imposed as a result of the Cl$^-$ gradient. This measured $E_{Cl^-}$ ideally should ideally equal the calculated $E_{Cl^-}$ determined by the Nernst equation. We used the measured $E_{Cl^-}$ (Cl$^-$, Br$^-$, I$^-$) values to determine a permselectivity sequence. Apparent differences between the measured $E_{Cl^-}$ and Nernst equation-calculated $E_{Cl^-}$ values are likely caused by ion permutation and accumulation in the unstirred layer closely adjacent to the membrane surface (37).

**Materials**—All biochemicals used were obtained from commercial sources and were of tissue culture grade or better.

**RESULTS**

Transfection and expression of P2X$_R$ in MTE18 cells followed by incubation of monolayers with 1 mM ATP and 10 μM ToPro-1-iodide (a membrane-impermeant dye that fluoresces when bound to DNA, $M_w = 645$) demonstrates efficient membrane permeabilization (Fig. 1). Although cells exposed to ATP for either 10 or 30 min showed distinct intracellular fluores-
exposed to serosal 1 mM ATP than to cells not permeabilized by 35030 activation by mucosal UTP revealed the presence of an apical similarly during the rest of the experimental protocol. Lumenal Cl− was successively diluted by replacement with a sodium gluconate solution to achieve a final luminal Cl− concentration of 68 mM, and 10 μM UTP was added to the lumenal solution (as indicated by the arrows). B, mean Cl− current responses in permeabilized (filled bars) and non-permeabilized (open bars) MTE18 monolayers. Gradient responses (left two bars) represent the total Isc, response following the final solution change. The Isc, in response to UTP (10 μM) addition following the imposition of the Cl− gradient is shown in the right two bars. Filled bars represent the mean current response of P2X7-R monolayers exposed to serosal ATP (permeabilized, n = 13), and open bars represent P2X7-R monolayers in the absence of serosal ATP (non-permeabilized, n = 14). Values represent mean and S.E. for each condition.

Conductance (Fig. 1, right two panels), P2X7-R-expressing cells not exposed to ATP showed no significant fluorescence beyond background levels (Fig. 1, left two panels). Control MTE18 cells or MTE18 cells expressing the control LISN vector, did not show any intracellular fluorescence in response to a 30-min exposure with 1 mM ATP (data not shown). We used this cell line (murine tracheal CFTR(−/−) epithelial cells, expressing P2X7-R) and selective permeabilization of the basolateral membrane to characterize CaCC resident in the apical membrane of airway epithelial cells.

Basolateral membrane permeabilization of murine tracheal epithelial cells followed by imposition of a Cl− gradient and activation by mucosal UTP revealed the presence of an apical membrane Cl− current (Fig. 2). Application of a cell to lumens Cl− gradient resulted in a greater response in monolayers exposed to serosal 1 mM ATP than to cells not permeabilized by ATP (Fig. 2B, 34.4 ± 9.2 versus 7.5 ± 2.4 μA/cm²). Importantly, addition of mucosal UTP to permeabilized cell monolayers was still capable of stimulating an increase in Isc, consistent with Cl− secretion (Fig. 2A, solid line trace, and Fig. 2B, right panel, filled bar). Similar responses to both an imposed Cl− gradient and mucosal UTP addition (ΔIsc = 54 ± 7.4 and 32.1 ± 8.9 μA/cm², n = 8, respectively) were observed in MTE18 (CF) monolayers permeabilized by S. aureus α-toxin. The magnitude of the Cl− secretory response to the purinergic agonist UTP was significantly greater in α-toxin-permeabilized MTE18 preparations than in α-toxin-permeabilized MTE7B (normal) preparations (32.1 ± 8.9 μA/cm², n = 8; 10.9 ± 3.7 μA/cm², n = 8, respectively, p < .001). Elevation of intracellular Ca2+ by inclusion of the ionophore, ionomycin (1 μM), showed a similar ability to stimulate Cl− secretion in permeabilized CF monolayers (25.8 ± 6.6 μA/cm², n = 8).

We have used several solution changes to verify that the observed current in MTE18-P2X7-R cells is a Cl− current. As shown above (Fig. 2) Cl− secretion (serosal to mucosal) is stimulated by imposition of a chemical gradient (i.e. lower Cl− concentration in the luminal solution). Reversal of this gradient, by decreasing the serosal Cl− concentration in permeabilized preparations, results in stimulation of “Cl− absorption” (mucosal to serosal) (Fig. 3). When the Cl− concentration in the luminal solution was maintained at 115 mM and the serosal solution was sequentially reduced, an absorptive Cl− current was recorded (Fig. 3A). The magnitude of the response was similar to the response observed for Cl− secretion (Fig. 2) but in the opposite direction (68.8 ± 7.4 versus 54.1 ± 8.2 μA/cm², respectively). Mucosal UTP was likewise able to augment this basal level of Cl− absorption by 18.2 ± 15.8 μA/cm². MTE18-P2X7-R cells that were not permeabilized (not exposed to serosal ATP) did not respond to the imposed absorptive gradient (data not shown).

We also studied the magnitude of the Cl− current when the serosal Cl− concentration was reduced to levels that approximate intracellular Cl− (~35 mM). We then imposed an out-
wardly directed Cl⁻ gradient by reducing luminal Cl⁻ to a similar ratio as previously studied (final mucosal Cl⁻ was diluted to a value of approximately 56% of the serosal concentration). Under these conditions, UTP was still capable of stimulating a characteristically similar Cl⁻ response, although the magnitude of the response was smaller than that observed with higher Cl⁻ concentrations (Fig. 3B).

The previous series of experiments involved dilution of the Cl⁻ concentration by substitution with the less permeant anion gluconate. This in effect results in a bi-ionic permselectivity relation, which provides an opportunity to determine the relative permeabilities of Cl⁻ and gluconate. We subsequently performed similar experiments in which Cl⁻ was substituted with Br⁻ or I⁻ (Fig. 4). Substitution of Cl⁻ with gluconate showed an increase in secretory Cl⁻ current (serosal to mucosal) as expected for a Cl⁻ dominated current. Both bromide and iodide substitution significantly attenuated the magnitude of this current (serosal to mucosal) as expected for a Cl⁻ dominated current. Both bromide and iodide substitution significantly attenuated the magnitude of this current, indicating that both of these halides, I⁻ and Br⁻ were more permeable than gluconate but less permeable than Cl⁻. Simply considering the Cl⁻ concentration on either side of the membrane, the Nernst equation would predict an equilibrium potential of ~13.6 mV. With gluconate as the counterion, we calculated an $E_{Cl^-}$ of ~9.8 mV. When bromide was substituted for Cl⁻, the calculated $E_{Cl^-}$ was ~6.7 mV, and when iodide was used as the counterion, $E_{Cl^-}$ was calculated to be ~3.8 mV. These increasing differences away from the Nernst equilibrium potential describe an anion selectivity sequence that is Cl⁻ > I⁻ > Br⁻ > gluconate.

Several putative Cl⁻ channel blockers were investigated for inhibition of UTP-stimulated Cl⁻ secretion in P2X₄-R-permeabilized MTE18 cells (Fig. 5). The most efficacious compounds appeared to be niflumic acid (NFA) (100 μM) and 5⁻nitrophenyl-propylbenzoate (NPPB; 100 μM), both of which inhibited approximately 90% of the CaCC-mediated current. The most routinely used Cl⁻ channel blocker, DIDS (100 μM), inhibited slightly more than 60% of the UTP-stimulated current, whereas glybenclamide (100 μM), a K⁺ channel blocker that has been shown to have efficacy against CPTP (38), blocked about 40% of the UTP-stimulated current. Finally, TS-TM calixarene (1 μM), a reported inhibitor specific for the outward rectifying Cl⁻ channel (36), the anti-estrogen tamoxifen (10 μM), shown to inhibit the human CIC2 channel (32), and diphenylamine-2-carboxylate (DPC) (100 μM) were essentially without effect (<10% inhibition) on the UTP-stimulated current.

**DISCUSSION**

We have previously shown that UTP, ionomycin, and thapsigargin are all capable of stimulating a Cl⁻ current in both CF and normal murine airway epithelial cells (33). In that study we demonstrated the presence of the Ca²⁺-activated Cl⁻ current and noted that the magnitude of the current was greater in CF (MTE18) than in normal (MTE7b) cells. In this study we have used permeabilization of the basolateral membrane to focus on an “apically isolated” preparation. Efficacy of permeabilization is apparent by the greater response to the imposed gradient observed in the permeabilized versus non-permeabilized preparations (compare filled with open bars in Fig. 2B). Dilutions of the luminal solution in non-permeabilized monolayers leads to only minor changes in current, but subsequent addition of UTP generates a large change in current. In permeabilized preparations, a large increase in current is observed in response to both changes in the Cl⁻ concentration, as well as the addition of UTP. Importantly, nucleotides ATP/UTP acting via the purinoreceptor, P2Y₂, have been shown to directly reduce the apical membrane resistance with no effect on the resistance of the tight junction (11). In our experimental design, imposition of a chemical gradient for Cl⁻ is likely to have effects on both the apical membrane conductance and the paracellular pathway, but application of UTP will only stimulate Cl⁻ secretion by activation of an apical membrane Cl⁻ channel. Thus, the response to UTP following permeabilization and imposition of the Cl⁻ gradient is convincing evidence for the existence of a Ca²⁺,activated apical membrane Cl⁻ channel. Furthermore, an increase in $I_{sc}$ in response to ionomycin is also confirmatory evidence that the Ca²⁺,mediated effects are the result of an apical Cl⁻ conductance rather than an effect on the paracellular pathway.

In an intact preparation, Ca²⁺ activation of K⁺ channels in the basolateral membrane likely plays an important role in maintaining the driving force necessary for Cl⁻ secretion. One obvious advantage of a permeabilized preparation is that we can eliminate the need for basolateral K⁺ channels by imposing a gradient by solution changes and thereby directly focus on the apical membrane. With these maneuvers we have shown that a Ca²⁺,activated Cl⁻ conductance is present in the apical
membrane of murine tracheal epithelial cells. Interestingly, UTP and ionomycin regulate this apical membrane Cl⁻ conductance even when Ca²⁺ is buffered to moderate levels (300 nM) by EGTA. This suggests that the accessory proteins necessary for regulation are not diazylated by permeabilization and that the mechanisms for intracellular Ca²⁺ release are also well preserved. Somewhat surprising, however, was the observation that UTP consistently activated this Cl⁻ conductance in the presence of 1 mM EGTA. EGTA is relatively slow in terms of buffering Ca²⁺ and may not be able to rapidly chelate release from local Ca²⁺ stores efficiently (39, 40). This experimental protocol allows us to buffer Ca²⁺ to physiological levels and preserves the ability to observe a UTP-mediated current response. These data are consistent with whole cell patch clamp studies that showed ATP-activated Cl⁻ currents in human airway epithelial cells in the presence of 10 mM EGTA (41). We have previously shown that UTP-stimulated currents in non-permeabilized MTE18 cells can be abolished by BAPTA-AM (33). Together, these results suggest that the UTP-stimulated current is Ca²⁺-dependent, but that released Ca²⁺ is capable of activating a target before it can be chelated by EGTA.

Heterologous expression of P2X₇-R in MTE18 cells provides a reliable, consistent, and rapid technique to generate an apically isolated preparation. Characteristically, the Cl⁻ secretory response to the imposed Cl⁻ gradient and to the luminal addition of UTP is similar to the responses observed in α-toxin permeabilized monolayers. Challenging P2X₇-R-expressing monolayers with ATP and the fluorescent DNA-intercalating agent, ToPro-1-iode, demonstrated that P2X₇-R effectively forms a pore sufficient for dialysis of the intracellular ion solution. These studies show that nearly every cell was expressing P2X₇-R and that receptor expression alone did not confer an increase in cell conductance, but rather that receptor occupancy by ATP was an absolute requirement for pore formation. Reversal of the Cl⁻ gradient confirmed that the preparation was permeabilized and also established that the current was Cl⁻-selective. Stable expression of P2X₇-R allows us an opportunity to determine the characteristics of the Ca²⁺-activated Cl⁻ conductance of the apical membrane in CF murine tracheal epithelial cells. This novel protocol for membrane permeabilization (transfection and activation of P2X₇-R) has the further advantage of serving as a self-contained control for permeabilization. By not exposing the cells to millimolar basolateral ATP, the cells function as an intact monolayer, thus the same monolayer preparation can be used for both non-permeabilized and permeabilized protocols.

We have characterized apical membrane CaCC in these preparations in terms of ion selectivity and inhibitor sensitivity. As mentioned earlier, several reports have provided differing characteristics for the CaCC. We believe that characterization in the CF murine airway apical membrane will provide the hallmark characteristics for this channel for subsequent whole cell and single channel analyses. Initial studies in P2X₇-R-permeabilized monolayers (Figs. 2 and 3) demonstrated a Cl⁻ current in response to a gradient that was generated by partial replacement of the mucosal Cl⁻ with the less permeant anion, gluconate. Although this fundamentally important experiment demonstrates the presence of the apical membrane Cl⁻ conductance, it also in fact serves as the first in a series of ion replacement studies. Although gluconate is often used as an “impermeant” anion, it is really only less permeant than the halide series. Therefore, a bi-ionic solution of Cl⁻ and gluconate can be evaluated for permeaselectivity based on the magnitude of the current response and a calculated equilibrium potential. As observed in Fig. 4, not surprisingly, Cl⁻ is more permeant than gluconate and results in a Cl⁻ current from serosal to mucosal solution. In contrast, when the mucosal Cl⁻ is partially replaced with iodide or bromide, we observed a smaller UTP-induced secretion and a greater shift from the ideal equilibrium potential, suggesting an ion series for this apical Cl⁻ conductance of Cl⁻ > I⁻ > Br⁻ > gluconate⁻. This anion selectivity sequence is somewhat similar to other reports of Ca²⁺-activated Cl⁻ channels (14, 41–43) but importantly differs from the selectivity sequence for CFTR (3, 4, 14, 42) or from the CLC superfamily of Cl⁻ channels (44).

Cl⁻ channel blockers were investigated to determine a “sensitivity sequence” for the CaCC channel. Pretreatment of permeabilized monolayers with the Cl⁻ channel blockers was used to determine a percentage inhibition of the UTP-mediated Cl⁻ current. The rank order of potency for channel inhibitors appears to be NFA, NPPB (~90%) > DIDS (~60%) > glybenclamide (~40%) > tamoxifen, TS-TM calixarene, and DPC (0–10%). The very low, nearly non-existent effect of DIDS-TM calixarene was consistent with previous studies that defined this compound as a specific inhibitor of the outward rectifying Cl⁻ channel (36). The moderate effect of glybenclamide on CaCC was a bit surprising, because this sulfonamide compound was thought to be fairly specific for K-ATF and CFTR channels (45, 46). In many studies DIDS is reported to be a highly effective inhibitor of Ca²⁺-activated Cl⁻ conductance (18–20, 41, 47), and although we observed moderate inhibition, it was not as effective as NFA. Higher doses of DIDS may generate higher levels of inhibition, but we are sensitive to the concerns of cross-linking that are associated with this compound. In our studies NFA was the most potent inhibitor of CaCC but like many Cl⁻ channel blockers lacks specificity, as it has been shown to also inhibit CFTR in human airways (47, 48). These studies serve to confirm the notion that Cl⁻ channel blockers are notoriously nonspecific, and inhibitor studies should be used simply as another characteristic to define the observed current. That is, an inhibitor order of potency or sensitivity sequence should serve an analogous function as a halide permeaselectivity sequence. The identity of the current should be defined by a combination of those characteristic sequences rather than any single effect.

Tamoxifen has recently been shown to have inhibitory effects against the human CIC2 channel (32). Interestingly, tamoxifen was without effect in our CF murine tracheal epithelial cells. Furthermore, we have not been able to detect the murine homologue of this channel, mCICα1, by Northern blot analysis in our CF murine tracheal epithelial cells, suggesting that the apical membrane Ca²⁺-activated Cl⁻ conductance is mediated by a thus far unidentified gene or protein.

We propose to utilize the activation, inhibition, and selectivity characteristics determined in this study for future experiments studying whole cell currents and single channel properties and ensure that the channel characteristics are consistent at each level of investigation. This systematic approach will result in the unambiguous identification of the murine airway CaCC localized to the apical membrane. Identification of CaCC along with a determination of regional distribution within the lung will permit us to develop strategies to activate CaCC and stimulate Cl⁻ and fluid secretion and thereby ameliorate the dehydration that is foremost in the pathology of cystic fibrosis.

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