In Situ Measurement of Airway Surface Liquid [K+] Using a Ratioable K+-sensitive Fluorescent Dye

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The airway surface liquid (ASL) is the thin fluid layer lining airway surface epithelial cells, whose volume and composition are tightly regulated and may be abnormal in cystic fibrosis (CF). We synthesized a two-color fluorescent dextran to measure ASL [K+]1, TAC-Lime-dextran-TMR, consisting of a green-fluorescing triazacryptand K+ ionophore-Bodipy conjugate, coupled to dextran, together with a red fluorescing tetramethylrhodamine reference chromophore. TAC-Lime-dextran-TMR fluorescence was K+-selective, increasing >4-fold with increasing [K+] from 0 to 40 mM. In well differentiated human airway epithelial cells, ASL [K+] was 20.8 ± 0.3 mM and decreased by inhibition of the Na+/K+ pump (ouabain), ENaC (amiloride), CF tranmembrane conductance regulator (CFTRinh-172), or K+ channels (TEA or XE991). ASL [K+] was increased by forskolin but not affected by Na+/K+2Cl− cotransporter inhibition (bumetanide). Functional and expression studies indicated the involvement of [K+] channels KCNQ1, KCNQ3, and KCNQ5 as determinants of ASL [K+]. [K+] in CF cultures was similar to that in non-CF cultures, suggesting that abnormal ASL [K+] is not a factor in CF lung disease. In intact airways, ASL [K+] was also well above extracellular [K+]: 22 ± 1 mM in pig trachea ex vivo and 16 ± 1 mM in mouse trachea in vivo. Our results provide the first noninvasive measurements of [K+] in the ASL and indicate the involvement of apical and basolateral membrane ion transporters in maintaining a high ASL [K+]. Abnormalities in ASL volume and/or composition are proposed to be important in the pathogenesis of cystic fibrosis (CF) lung disease. The ASL is formed by a combination of fluid secretion by airway submucosal glands, convective fluid transport up the airway tree, and water/ion transport by airway surface epithelial cells, the latter likely playing a key role in active regulation of ASL volume and ionic composition.

Although early data suggested that salt concentration in the ASL is low in normal airways (4), the current view is that the ASL is approximately isotonic in both normal and CF airways (5–7). A concern with older measurements of ASL composition involving fluid collection by filter paper or microcapillaries is perturbation of the airway surface and the sampling, by capillary forces, of more fluid than contained in the very thin (tens of microns) ASL layer. Studies using ion-sensitive microelectrodes, although technically demanding and requiring direct contact with the ASL, provided evidence for a nearly isotonic ASL (5). Our laboratory developed ratioable fluorescent dyes to measure ASL [Na+] and [Cl−], in which the ASL was fluorescently stained for determination of ion concentrations by ratio imaging microscopy (7). ASL salt concentration ([Na+] and [Cl−]) was found to be approximately isotonic in airway epithelial cell cultures, mouse trachea and small airways, and ex vivo human airways, without differences in CFTR deficiency (7, 8). We also found the ASL to be approximately isomolar with serum using fluorescent, osmotically sensitive liposomes (9).

Relatively little is known about ASL potassium concentration ([K+]i) or its regulation. As diagrammed in Fig. 1A, transcellular transport of K+ is believed to involve the coordinated activities of a Na+/K+ pump, Na+/K+/2Cl− cotransporter, and K+ channel(s) at the basolateral membrane and a H+/K+ pump and K+ channel(s) at the apical membrane. The airway epithelium also has significant paracellular ion permeability. There is evidence for functional expression of several types of K+ channels in cell lines derived from airways/lung, including Ca2+-activated, cAMP-activated, and voltage-activated K+ channels (10–13). Steady state [K+] in a stationary ASL (without fluid convection) should depend on the activities of cell membrane K+ pumps and ion transporters, as well as non-K+ ion channels, such as CFTR and ENaC, which are involved in establishing membrane potentials and thus the electrochemical driving forces for K+ transport.

The purpose of this study was to develop a noninvasive fluorescence method to measure [K+]i in the ASL and to establish the major determinants of [K+] regulation. Following several years of synthetic chemistry, we developed a series of water-soluble K+ sensors, the first being TAC-Red, in which K+ bind-
ing to a triazacyclpentane (TAC) K⁺ ionophore results in fluorescence enhancement of a conjugated xanthylum chromophore by a charge transfer quenching mechanism (14). TAC-Red was used to follow K⁺ waves in the extracellular space in brain in a neuroexcitation model of cortical spreading depression. Second generation K⁺ sensors of different colors, TAC-Crimson (15) and TAC-Lime (16), work by a similar K⁺-sensing mechanism but utilize different chromophores. These indicators are selective for K⁺ under physiological conditions and respond to changes in [K⁺] in milliseconds or less (15). For the measurements here, we synthesized a dextran conjugate containing TAC-Lime, which has K⁺-sensitive green fluorescence, and tetramethylrhodamine, a reference chromophore with K⁺-insensitive red fluorescence. The indicator allowed technically straightforward determination of ASL [K⁺] in cell culture and in vivo models by fluorescence ratio imaging.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Dextran-conjugated Fluorescent K⁺ Indicator**—The four-step synthesis of the disuccinimidyl ester of TAC-Lime (TAC-Lime-DiSE; Fig. 2A) from TAC-aldehyde was reported previously (16). Synthesis of the K⁺-binding TAC-aldehyde involves 17 steps (17). Conjugation of TAC-Lime-DiSE with amino dextran (10 kDa; Molecular Probes, 4 mol free amine/mol dextran) was accomplished by reacting for 12 h three molar equivalents of TAC-Lime-DiSE with 1 molar equivalent amino dextran in an aqueous solution buffered at pH 7.4. Purification was accomplished by repetitive precipitation in methanol. Subsequent reaction with 1 molar equivalent of tetramethylrhodamine-succinimidyl ester (TMR-SE) was accomplished using the same conditions to yield TLT-dex.

**Perfusion Chambers**—A stainless steel perfusion chamber was constructed to accommodate 12-mm-diameter cell culture inserts (Fig. 1B). The chamber was held in a PDMI-2 microincubator (Harvard Apparatus, Holliston, MA) maintained at 37 °C. The perfusates were heated by in-line heater and delivered at 1 ml/min by gravity to accomplish solution exchange in under 20 s. The chamber was surrounded by an enclosure containing air or air/5% CO₂, which was humidified using a Fisher-Milligan gas washer (Fisher). A second perfusion chamber was constructed for pig trachea, in which fragments were impaled with four small pins and covered with a stainless steel plate having a central, 10-mm circular opening to visualize the fluorescently stained ASL.

**Cell Culture**—Human airway bronchial epithelial cells (non-CF and CF) were cultured at an air-liquid interface at 37 °C in 5% CO₂, 95% air as described previously (18). Briefly, the cells were dissociated by enzymatic digestion and suspended in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 5% fetal calf serum (Hyclone, Logan, UT), gentamicin (50 μg/ml), penicillin (100 units/ml), streptomycin (1 mg/ml), and fungizone (2.5 μg/ml) and seeded at a density of 10⁴ cells/cm² onto 12-mm Transwell polycarbonate inserts (0.4-μm pore size; Costar, Corning, NY) overlaid with a thin coat (15 μg/cm²) of human placental collag en (Sigma-Aldrich). The next day the cells were rinsed with phosphate-buffered saline, and the medium was replaced with ALI medium (19) containing gentamicin, penicillin, and streptomycin at the concentrations given above. The medium was changed every 3 days. Transepithelial resistance was measured using an epithelial volt ohm meter (World Precision Instruments, Sarasota, FL). The cultures were used 21 days after plating, at which time full mucociliary differentiation was confirmed by histology, resistance was 1008 ± 35 and 976 ± 85 Ω·cm² in normal and CF cultures, respectively, and a thin ASL film was seen. The range of amiloride-sensitive currents was 2–20 μA/cm², and CFTR (forskolin)- and CaCC (ATP)-dependent currents were 15–50 μA/cm².

**Fluorescent Staining of ASL**—Small quantities of finely ground TLT-dex or rhodamine B-dextran (10 kDa; Molecular Probes) were applied to the ASL as dry powders or after dispersion by probe sonication in low boiling point perfluorocarbon (compound FC-72; boiling point, 56 °C; 3M Company, St. Paul, MN) as described (7). TLT-dex was fully dissolved in ASL after 1 h of incubation at 37 °C in a 5% CO₂ incubator. The concentration of TLT-dex in the ASL was ~50 μM based on comparative intensity measurements using solution standards. Control fluorescence measurements showed that added TLT-dex could be fully washed off, indicating little endocytosis or surface adhesion.

**Microscopy**—Fluorescence was measured using a Nikon SMZ stereo-zoom epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a P-HR Plan Apo 1.6× objective lens (working distance, 24 mm), Hamamatsu ORCA-ER CCD camera, and custom filter sets for TAC-Lime and TMR (Chroma, Rockingham, VT). Green/red image pairs, each 1-s acquisitions, were obtained from three to five different spots/filter at each time point. The illumination light was shuttered off between image acquisitions. There was less than 1% dye photobleaching after 30 image acquisitions. The fluorescence signal was generally 10-fold greater than background, with <15% variation in ratios in different fields of a cell culture.

**Measurements in Trachea in Living Mice**—Mice (25–35 g of body weight) were anesthetized with avertin (200 mg/kg), and body temperature was maintained at 37–38 °C using a heating pad and rectal temperature probe. Following a midline neck incision, a 1–1.5-cm length of trachea was bluntly isolated for direct visualization. As done previously for other dyes (7), the ASL was stained by direct disposition of TLT-dex onto the trachea using a micrcatheter inserted via the mouth. The mice breathed room air spontaneously. The trachea was stabilized for imaging. Fluorescence images of the trachea were obtained using the Nikon SMZ microscope as described above, where the brightly dye-stained ASL was visualized through the translucent tracheal wall. The images for background subtraction were obtained prior to dye instillation. The mice were sacrificed by pentobarbital overdose at the completion of the measurements. Mouse protocols were approved by the University of California, San Francisco, Committee on Animal Research.

**Measurements of ASL Thickness**—ASL thickness was measured using a laser scanning confocal microscope (Nikon, Tokyo, Japan), equipped with a water immersion objective lens (60×; numerical aperture, 1.2; working distance, 270 μm). For these measurements the ASL was covered with high boiling point perfluorocarbon FC70 (3M Company) to prevent evaporation and to allow immersion of the objective lens (refractive
index of FC70 is 1.303, similar to that of water 1.3). A stack of 100 images (1 μm apart) was acquired over 40–60 s for reconstruction of x-z and y-z images to determine ASL thickness. To measure ASL thickness in cell cultures, fine solid particles of TLT-dex were deposited onto the ASL. z scanning was done 2 h after ASL staining, with FC-70 covering the cells/ASL. For measurements in pig trachea, pig tracheas were obtained from Pork Power Farms (Turlock, CA) and were generally delivered to the lab on ice within 12 h after animal sacrifice. Small fragments of tracheal mucosa was rinsed with phosphate-buffered saline and mounted in the perfusion chamber. The fragment in the perfusion chamber was incubated for 5 h at 37 °C in the CO2 incubator and then stained with dye as done for the cell cultures. ASL images were obtained 1 h after staining. The accuracy of ASL thickness determination was confirmed by measurements of known fluid layers of 5–50 μm between two cover glasses (with objective immersed in FC70 on top of the cover glass). Reported ASL thicknesses are an average from three samples, with at least five different areas averaged for each sample.

Electrophysiology—Human airway bronchial epithelial cells grown on permeable supports (Snapwell; 1.12-cm² surface area) were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA). The cells were bathed for a 30-min stabilization period in HCO3-/H11002-buffered solution and aerated with 95% O2, 5% CO2 at 37 °C. Transepithilial potential difference (PD) and short circuit current (Isc) were measured using EV9400 multi-channel V/I clamp (World Precision Instruments, Sarasota, FL) and recorded using PowerLab/8sp (AD Instruments Pty Ltd., Castle Hill, Australia). For measurement of PD, electrode zero offset was determined before and after each recording. PD and Isc were recorded continuously with agonists/inhibitors added at specified times. For Isc measurement of apical K⁺ conductance, the basolateral membrane was permeabilized with amphotericin B (20 μM), valinomycin (10 μM), and nigericin (10 μM), and a K⁺ gradient was applied; the apical solution contained 140 mM sodium gluconate, 5 mM potassium gluconate, 1 mM MgSO4, 1 mM CaSO4, 10 mM D-glucose, and 10 mM HEPES (pH 7.4) and in the basolateral solution 140 mM sodium gluconate was replaced by potassium gluconate. Apical ENaC was inhibited by 10 μM amiloride. To measure basolateral K⁺ conductance, the apical membrane was permeabilized with 10 μM amphotericin B, and a K⁺ gradient was applied. Transepithelial resistance was calculated using Ohm’s law from current changes in response to 10-mV pulses.

Reverse Transcription (RT)-PCR—Expression of messenger RNA (mRNA) encoding KCNQ1–5, KCN1–4, KCN1–4, and ATP1AL1 was determined by RT-PCR in primary cultured and freshly isolated human bronchial epithelial cells. Total RNA was extracted by using a TRIzol solution (Invitrogen) and was reverse-transcribed by using random hexamer primers and a ribonuclease H reverse transcriptase (Invitrogen). The complementary DNA was amplified with specific primers and Taq polymerase (Invitrogen). The primer sequences are provided in Table 1.

Immunofluorescence—Immunostaining of human airway epithelial monolayers was performed using antibodies against the KCNQ1 (sc-20816; Santa Cruz Biotechnology), KCNQ3 (AB5597; Chemicon), KCNQ5 (AB5599; Chemicon), and Na⁺/K⁺-ATPase α (sc-58628; Santa Cruz Biotechnology). Briefly, the monolayers were fixed and permeabilized by incubation in cold methanol for 10 min at −20 °C. Nonspecific binding was
**RESULTS**

Most of the ASL K\(^+\) measurements reported here were done on well differentiated primary cultures of airway epithelial cells from normal (non-CF) humans. Fig. 1A shows the principle plasma membrane transporters expressed on these cells. The cell cultures used for measurements formed a tight epithelium with transepithelial resistance 1008 ± 35 Ω cm\(^2\) (S.E., \(n = 12\)) and contained little apical surface fluid. The perfusion chamber used for K\(^+\) measurements is shown schematically in Fig. 1B. The insert containing the cultured airway cells was placed in the chamber with the bottom surface perfused continuously and the top (apical) surface exposed for fluorescent dye staining and imaging. The temperature was maintained at 37 °C for most measurements, and the cells were bathed in a humidified atmosphere.

The cell cultures expressed ENaC Na\(^+\) channels and CFTR Cl\(^-\) channels, with electrophysiological responses similar to those reported previously (22, 23). Fig. 1C shows a representative short circuit current measurement in which ENaC inhibition by amiloride reduced current by 2.7 ± 0.5 μA/cm\(^2\) (\(n = 9\)). CFTR activation by forskolin increased short circuit current, which was inhibited by the thiazolidinone CFTR inhibitor CFTRinh-172 (20). The addition of ATP, which transiently elevates cytoplasmic Ca\(^2+\), produced a transient increase in short circuit current caused by activation of Ca\(^2+\)-dependent Cl\(^-\) and K\(^+\) channels. This current was inhibited by CaCCinh-A01, an inhibitor of calcium-activated Cl\(^-\) channels (21). Open circuit measurements of transepithelial PD were also done on these cells to evaluate electrochemical driving forces for analysis of ASL K\(^+\) data. Fig. 1D shows a base-line PD of −6.7 ± 0.5 mV (apical side negative, \(n = 9\)), which was relatively insensitive to omeprazole and amiloride but sensitive to modulation of CFTR and calcium-activated Cl\(^-\) channels. As expected, omeprain, an inhibitor of the Na\(^+\)/K\(^+\)-ATPase, abolished most of the PD.

Measurements of K\(^+\) were done by ratio imaging using a dextran conjugate containing TAC-Lime, whose green fluorescence is K\(^+\)-sensitive, and TMR, whose red fluorescence is K\(^+\)-insensitive. Fig. 2A shows the route for synthesis of the dual wavelength K\(^+\) indicator TAC-Lime-TMR-dextran (TLT-dex). TAC-Lime-SE, synthesized in four steps from TAC-aldehyde, together with TMR. Fig. 2B shows the dual wavelength emissions spectra of TLT-dex in solutions containing different K\(^+\) concentrations.
**Airway Surface Liquid \([K^+]\)**

![Diagram](image)

**FIGURE 2. Synthesis and characterization of two-color \(K^+\)-sensitive fluorescent indicator, TLT-dex.** A, synthesis of TLT-dex, a dextran conjugate containing TAC-Lime and TMR, was accomplished by reaction of 3 mol of Tac-Lime-DISe with 1 mol of amino dextran followed by reaction with 1 mol of TMR-SE (see "Experimental Procedures"). B, fluorescence emission spectra of TLT-dex in solutions containing indicated \([K^+]\). Excitation wavelength was 490 nm. Inset, TLT-dex green-to-red fluorescence \(F_{\text{green}}/F_{\text{red}}\) as a function of \(pH\) in solutions containing 30 mM \(K^+\). C, dependence of \(F_{\text{green}}/F_{\text{red}}\) on \([K^+]\) at \(pH 7.4\) as measured by fluorescence microscopy (S.E. \(n = 4\)).

**FIGURE 3. ASL \([K^+]\) in well differentiated HBE cells.** A, fluorescence images of ASL staining by TLT-dex. Pseudocolored ratio image (computed after background subtraction) shown on the right. Bar, 200 \(\mu\)m. B, \(F_{\text{green}}/F_{\text{red}}\) (left \(y\) axis) and deduced \([K^+]\) (right \(y\) axis) measured over time in cells perfused continuously with \(HCO_3^-\)containing buffer in the absence or following basolateral addition of 100 \(\mu\)M ouabain, as indicated. The ASL was stained with TLT-dex. The data are representative of three sets of experiments; the error bars represent S.E. from ratio measurements done on five different spots on filters. C, \(F_{\text{green}}/F_{\text{red}}\) measurements done following addition of ionophores (nigericin, 10 \(\mu\)M; valinomycin, 10 \(\mu\)M) and ouabain (100 \(\mu\)M) and perfusion with solutions containing indicated \([K^+]\). D, sensitivity of ASL \([K^+]\) to perfusate \([K^+]\). \(F_{\text{green}}/F_{\text{red}}\) measured in untreated cells following indicated changes in perfusate \([K^+]\).

In these studies, \([Na^+] + [K^+]\) was kept constant; as found previously, the trizacryptand chromophore is highly selective for \(K^+\) versus \(Na^+\) (14). TLT-dex fluorescence (maximum at 533 nm) increased with increasing \([K^+]\), whereas TMR fluorescence (maximum at 582 nm) was not sensitive to \([K^+]\), although the tail of the TAC-Lime green fluorescence produced a small \(K^+\)-dependent fluorescence increase at 584 nm. Fig. 2B (inset) shows that TLT-dex green-to-red fluorescence ratio \(F_{\text{green}}/F_{\text{red}}\) is not sensitive to \(pH\) in the range 4.5–8.5. Also, \(F_{\text{green}}/F_{\text{red}}\) was not sensitive to \(Ca^{2+}, Cl^-\), or albumin but was sensitive to the nonphysiologic calcium \(Rb^+\), which is of similar size/charge to \(K^+\) (not shown). Fig. 2C shows a curvilinear relationship between \(F_{\text{green}}/F_{\text{red}}\) and \([K^+]\) as measured using the fluorescence microscope, with good sensitivity over a wide concentration range appropriate for measurements of \([K^+]\) in extracellular space compartments such as the ASL.

For \([K^+]\) measurements, the ASL was stained with TLT-dex without the addition of aqueous volume. Image pairs of green and red fluorescence were obtained at three to five different spots on a filter for each time point. Fig. 3A shows fluorescence images of the TLT-dex-stained ASL in HBE cells together with a pseudocolored ratio image. The ratio image shows similar \(F_{\text{green}}/F_{\text{red}}\) ratios throughout the stained ASL, with variance of <10%. Fig. 3B shows \(F_{\text{green}}/F_{\text{red}}\) and deduced \([K^+]\) in control and ouabain-treated cell cultures. \(F_{\text{green}}/F_{\text{red}}\) in untreated perfused cell cultures was generally stable over >60 min. Average \(F_{\text{green}}/F_{\text{red}}\) for measurements on human bronchial epithelial cell cultures was 0.318 ± 0.002 (S.E., \(n = 45\)), corresponding to \([K^+]\) of 20.8 ± 0.3 mM, substantially greater than perfusate \([K^+]\) of ~5 mM. \(F_{\text{green}}/F_{\text{red}}\) ratios were quite reproducible in different regions of each filter. Inhibition of the \(Na^+\)/\(K^+\) pump by ouabain, which largely dissipates cellular electrochemical driving forces, produced a slow decline in \(F_{\text{green}}/F_{\text{red}}\) to ~0.18 at 2 h, corresponding to \([K^+]\) of ~7 mM.

Fig. 3C shows an in situ calibration where \(F_{\text{green}}/F_{\text{red}}\) was measured following changes in perfusate \([K^+]\) in the presence of \(K^+\) ionophores (valinomycin and nigericin) and ouabain,
changes in perfusate $[K^+]$ from 2 to 8 mM (Fig. 3D), suggesting active regulation of ASL $[K^+]$ involving cell membrane ion transporters.

The determinants of ASL $[K^+]$ were investigated using a series of modulators of membrane transporters. Fig. 4A shows representative time courses of $F_{\text{green}}/F_{\text{red}}$ and deduced $[K^+]$ for cultures treated with ATP, TEA (a nonspecific $K^+$ channel blocker), forskolin, and CFTRinh-172. ATP, TEA, and CFTRinh-172 produced significant reductions in ASL $[K^+]$, whereas forskolin produced an increase in ASL $[K^+]$. As supported by further studies, below, the reduced ASL $[K^+]$ with CFTR inhibition (by CFTRinh-172) and the increased ASL $[K^+]$ with CFTR activation (forskolin) are anticipated consequences of altered apical membrane $Cl^-$ secretion and membrane potential. The reduced ASL $[K^+]$ with ATP is a multifactorial effect, involving a large, basolateral $Ca^{2+}$-activated $K^+$ conductance. The reduced ASL $[K^+]$ with TEA under basal conditions is primarily due to reduced apical membrane $K^+$ conductance.

Fig. 4B summarizes ASL $[K^+]$ measured before and at 2 h after the addition of indicated agonists/inhibitors of each of the major airway epithelial cell ion transporters. No significant effect was found for bumetanide inhibition of $Na^+/K^+/2Cl^-$ cotransporter, DIOA inhibition of the $K^+/Cl^-$ cotransporter, and bafilomycin A1 inhibition of the vacuolar-type H$^+$-ATPase. Although the two latter transporters have not been reported in airway epithelia, we felt that functional measurements were important in ruling out their involvement in the maintenance of a high ASL $[K^+]$. Reduced ASL $[K^+]$ was found for amiloride inhibition of ENaC, CFTRinh-172 which should approximately equalize ASL and perfusate $[K^+]$.

$F_{\text{green}}/F_{\text{red}}$ was sensitive to perfusate $[K^+]$ in the presence of ionophores/ouabain, with similar $F_{\text{green}}/F_{\text{red}}$ versus $[K^+]$ as measured in calibration solutions. To investigate whether ASL $[K^+]$ resists large changes in perfusate $[K^+]$, $F_{\text{green}}/F_{\text{red}}$ was measured in untreated cells following changes in perfusate $[K^+]$. ASL $[K^+]$ was changed from 13 to 20.6 mM following inhibition of CFTR, ouabain inhibition of the $Na^+/K^+$ pump, omeprazole inhibition of the $H^+/K^+$ pump, and ATP activation of CaCCs. Increased ASL $[K^+]$ was found following forskolin, which activates CFTR as well as cAMP-regulated $K^+$ channel(s). Open circuit PDs were measured for each of these maneuvers in parallel experiments. ATP and forskolin increased basal PD (lumen negative) by 2.6 and 3.1 mV (mean

**FIGURE 4. Determinants of ASL $[K^+]$ in HBE cells.** A, $F_{\text{green}}/F_{\text{red}}$ and deduced $[K^+]$ measured over time following basolateral addition of 100 $\mu$M ATP, 10 mM TEA, 10 $\mu$M forskolin, or 20 $\mu$M CFTRinh-172. B, summary of ASL $[K^+]$ (S.E., four to six measurements on different filters) measured at 0 and 120 min after incubation with indicated compounds. The compounds were added to basolateral solution. The concentrations used were: amiloride (500 $\mu$M), bumetanide (100 $\mu$M), DIOA (100 $\mu$M), CFTRinh-172 (20 $\mu$M), ouabain (100 $\mu$M), TEA (10 mM), XE991 (100 $\mu$M), omeprazole (300 $\mu$M), ATP (100 $\mu$M), forskolin (10 $\mu$M), and bafilomycin A1 (100 nM). *, $p < 0.05$ compared with 0 h.

**FIGURE 5. Effect of $K^+$ channel blockers on basal, cAMP-stimulated and $Ca^{2+}$-stimulated $I_{sc}$ in HBE cells.** A, basal $I_{sc}$, amiloride (10 $\mu$M) was added to luminal (L) and basolateral (B) solutions. XE991 (100 $\mu$M), TEA (10 mM), and Ba$^{2+}$ (5 mM) were added to luminal and basolateral solutions as indicated. Right panel, reduction of basal $I_{sc}$ by luminal and basolateral Ba$^{2+}$ (S.E., three measurements on different filters). C and D, $I_{sc}$ measured after cAMP simulation (forskolin, 10 $\mu$M) and $Ca^{2+}$ stimulation (ATP, 100 $\mu$M). The percentage of inhibition of cAMP- and $Ca^{2+}$-stimulated $I_{sc}$ by luminal and basolateral Ba$^{2+}$ application summarized in bar graphs (S.E., three measurements on different filters).
Airway Surface Liquid \([K^+]\)

\(\Delta PD, n = 2 – 3, \text{ S.D.} \sim 1 \text{ mV})\), respectively. Amiloride, ouabain, CFTR\(_{\text{inh}}\)-172, XE991, TEA, bumetanide, and DIOA decreased basal PD by 1.2, 6.6, 5.6, 6.4, 1.2, 2.2, and 2.4 \text{ mV}, respectively. Bafilomycin A and omeprazole did not alter basal PD significantly. These relatively modest effects on PD support the conclusion that transcellular rather than paracellular factors are primarily responsible for the high ASL \([K^+]\) in our system.

Because of their likely central involvement in maintaining high ASL \([K^+]\), further studies were done to characterize \(K^+\) channels in the human airway epithelial cell cultures. We studied the effects of \(K^+\) channel inhibitors on \(I_{\text{sc}}\) in intact HBE monolayers in which ENaC was inhibited by amiloride, under basal conditions, and after cAMP and \(Ca^{2+}\) stimulation. Fig. 5A shows reduced basal \(I_{\text{sc}}\) by luminal application of XE991, an inhibitor of KCNQ \(K^+\) channels, and the nonselective \(K^+\) channel blockers, TEA and \(Ba^{2+}\). Basal \(I_{\text{sc}}\) was inhibited by luminal and basolateral application of \(Ba^{2+}\) in a manner that did not depend on the order of addition (Fig. 5B). Luminal and basolateral application of \(Ba^{2+}\) decreased basal \(I_{\text{sc}}\) by 0.73 \pm 0.09 and 6.2 \pm 1.1 \mu A/cm\(^2\), respectively. Luminal and basolateral application of \(Ba^{2+}\) inhibited cAMP-stimulated (by forskolin) \(I_{\text{sc}}\) by 8.8 \pm 0.6 and 89 \pm 1\% (Fig. 5C) and \(Ca^{2+}\)-stimulated \(I_{\text{sc}}\) (by ATP) by 9.0 \pm 0.8 and 79 \pm 3\% (Fig. 5D).

It has been suggested that KCNQ, a voltage-gated \(K^+\) channel, with six membrane-spanning domains, and its regulatory subunits, KCNE, are involved in cAMP-activated anion secretion in airway epithelia, and that KCNN4, an intermediate/small conductance \(K^+\) channel, is involved in \(Ca^{2+}\)-activated anion secretion (13, 24–26). The KCNQ1-specific blocker, chromanol 293B, and the general KCNQ blocker, XE991, were used to investigate KCNQ channel inhibition on cAMP- and \(Ca^{2+}\)-stimulated \(I_{\text{sc}}\) in HBE cells. Fig. 6A shows that luminal and basolateral application of chromanol 293B partially inhibited cAMP-stimulated \(I_{\text{sc}}\) with nearly complete inhibition by XE991. Fig. 6B shows that XE991 pretreatment inhibited forskolin-induced peak \(I_{\text{sc}}\) by 96.8 \pm 0.2\%. \(Ca^{2+}\)-stimulated peak \(I_{\text{sc}}\) was blocked by 70 \pm 5\% by XE991 pretreatment. These results indicate the involvement of KCNQ channels in cAMP- and \(Ca^{2+}\)-stimulated anion secretion.

To investigate apical and basolateral membrane \(K^+\) conductances, membranes of HBE monolayers were individually permeabilized by 30 min of incubation with amphotericin B in the presence of a \(K^+\) gradient (see “Experimental Procedures”). Fig. 7A shows apical membrane \(K^+\) conductance. Amphotericin B (20 \mu M), valinomycin (a \(K^+\) ionophore, 10 \mu M), and nigericin (a \(K^+/H^+\)-antiporter, 10 \mu M) were used to ensure basolateral membrane permeabilization. Apical ENaC was inhibited by amiloride. Application of XE991 inhibited basal \(I_{\text{sc}}\) by 0.9 \pm 0.2 \mu A/cm\(^2\). cAMP elevation by forskolin slightly increased apical membrane \(K^+\) conductance (0.8 \pm 0.1 \mu A/cm\(^2\)). Fig. 7B shows basolateral membrane \(K^+\) conductance. Application of 1-ethyl-2-benzimidazolinone produced a large increase in \(I_{\text{sc}}\) that was inhibited by 30 \mu M clotrimazole, an inhibitor of \(Ca^{2+}\)-activated \(K^+\) channels. Forskolin, in contrast, produced only a small increase in \(I_{\text{sc}}\) that was sensitive to XE991.

Prior data suggest that some KCNQ channels, including KCNQ1, KCNE2, KCNE3, KCNN4, and ATP1A1, a nongastric \(H^+\), \(K^+\)-ATPase, are expressed in human airway epithelia (13, 24, 27). RT-PCR was done to test for expression of transcripts encoding KCNQ1–5, KCNE1–4, KCNN1–4, and ATP1A1. Fig. 7C shows expression of KCNQ1, KCNQ3, KCNQ5, KCNE1–4, KCNN3–4, and ATP1A1 mRNA in both primary cultures and freshly isolated HBE cells. KCNN2 mRNA was seen only in freshly isolated HBE cells. KCNQ2, KCNQ4, and KCNN1 mRNA were not expressed in cultures of freshly isolated HBE cells. In contrast, Calu-3 cells did not express KCNE1 mRNA (24). We confirmed KCNQ1 expression in HBE cells by RT-PCR using two different sets of KCNQ1-specific primers and cDNAs from three human subjects (data not shown).

Membrane localization of KCNQ1, KCNQ3, and KCNQ5 was determined in the cultured HBE cells by confocal immunofluorescence microscopy (Fig. 7D). Immunostaining revealed colocalization of KCNQ1 with \(Na^+/K^+\)-ATPase, a basolateral membrane marker, as well as expression on the apical membrane. KCNQ3 and KCNQ5 were detected only in the apical membrane.

CFTR activity was further characterized to understand effects of CFTR inhibition and activation on ASL \([K^+]\). Application of CFTR\(_{\text{inh}}\)-172 decreased basal \(I_{\text{sc}}\) by 2.8 \pm 0.5 \mu A/cm\(^2\) (Fig. 8A) and basal PD by 5.6 \pm 2.0 \text{ mV} (data not shown), indi-
FIGURE 7. K⁺ channel identification in HBE cells by short circuit current analysis, RT-PCR, and confocal immunofluorescence microscopy. A, apical K⁺ conductance measured after basolateral membrane permeabilization by amphotericin B (20 μM), valinomycin (10 μM) and nigericin (10 μM) and in the presence of indicated apical and basolateral solution K⁺. XE991 (100 μM) and forskolin (10 μM) were added as indicated. Basolateral K⁺ conductance was measured after apical membrane permeabilization by amphotericin B and in the presence of indicated K⁺ gradient. 1-Ethyl-2-benzimidazolinone and forskolin were applied to activate basolateral Ca²⁺- and cAMP-activated K⁺ channels. Clotrimazole (30 μM), an inhibitor of Ca²⁺-activated K⁺ channels, and XE991 were added as indicated. B, RT-PCR, using primers specific for KCNQ1–5, KCNE1–4, KCN1–4, and ATP1A1, done using as template HBE primary cell cultures (top panel) and freshly isolated human bronchial epithelial cells (bottom panel). The data are representative of three sets of amplifications. D, Immunolocalization of KCNQ1, KCNQ3, KCNQ5 (red), and Na⁺/K⁺-ATPase (green). The upper panels show x-y confocal images just below the cell apical surface, with x-z images shown in the lower panels. KCNQ1 immunoblot is shown in the left panels. The arrowheads indicate apical (ap) and basol (ba) membranes. Bar, 10 μm.

FIGURE 8. Role of CFTR in ASL [K⁺]. A, basal CFTR activity in non-CF HBE cells was measured by addition of 10 μM CFTRinh-172. B, ASL was stained with rhodamine B-dextran (10 kDa), and cells were stained with CFDA-SE Cell Tracer (10 μM). ASL thickness measured under basal conditions and 2 h after stimulation by basolateral application of ATP (100 μM) or forskolin/3-isobutyl-1-methylxanthine (20 μM/100 μM). Bar, 20 μm. C, short circuit current in CF human epithelial cell culture in response to additions of amiloride (10 μM), forskolin (10 μM), CFTRinh-172 (10 μM), ATP (100 μM), XE991 (100 μM), and ouabain (100 μM). D, ASL [K⁺] was measured in CF cultures before and at 2 h after incubation with ATP (100 μM). Measurements in A–D are representative of three to five studies on different cultures. *, p < 0.05; **, p < 0.01 compared with control.

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with CFDA-SE Cell Tracer. ASL thickness under basal conditions was 7.7 ± 0.4 μm, similar to previously reported values (2, 28). ATP and forskolin/3-isobutyl-1-methylxanthine increased ASL thickness to 20.7 ± 1.0 and 26.3 ± 1.8 μm, respectively.

Measurements were done on human CF airway epithelial cell cultures to determine whether ASL [K⁺] is abnormal in CF. The short circuit current measurement on a CF culture in Fig. 8C shows robust amiloride and ATP effects, with little or no effect of forskolin or CFTRinh-172, as expected. The ATP-induced current was inhibited by XE991 and ouabain. Fig. 8D summarizes ASL [K⁺] measurements in CF tracheal epithelial cell cultures. ASL [K⁺] in CF was similar to that in non-CF cell cultures. As found in the non-CF cultures, ASL [K⁺] was reduced by ATP.

Lastly, measurements of ASL [K⁺] were done in intact trachea using two model systems: ex vivo porcine trachea and in vivo mouse trachea. Fragments of viable pig tracheas were prepared for measurements by removal of the cartilage and serosal tissues and mounted in a custom perfusion chamber (Fig. 9A, top panel) in which the serosal surface was perfused continuously and the mucosal surface was exposed for dye staining and fluorescence imaging. Fig. 9A (bottom panel) shows a photograph of the perfusion chamber containing a fragment of pig trachea. F green/F red measurements were made following ~5 h of incubation at 37°C to stabilize ASL properties. Fig. 9B (top panel) shows z scanning confocal images of the TLT-dextran-stained ASL, indicating quite uniform [K⁺] throughout the ASL in pig trachea. Fig. 9B (bottom panel) shows stable F green/F red. Average F green/F red was 0.326 ± 0.004 (in HCO₃⁻-free buffer) and 0.328 ± 0.006 (in HCO₃⁻-containing buffer), corresponding to ASL [K⁺] of 21.8 ± 0.5 and 22.0 ± 0.8 mM (S.E., n = 7), respectively (Fig. 9D). Incubation with ouabain (100 μM) reduced ASL [K⁺] in pig trachea by >5 mM (data not shown).
Airway Surface Liquid [K⁺]

Measurements of ASL [K⁺] were also made in anesthetized, spontaneously breathing mice following TLT-dextran staining of the ASL. The mouse trachea was exposed by blunt dissection for direct visualization. TLT-dextran was added through the mouth using a microcatheter, as done previously for Na⁺ and pH-sensing dyes (7). Sufficient dye staining was found to enable fluorescence measurements through the thin translucent wall of the mouse trachea. Fig. 9C shows a bright light photograph of the mouse trachea, along with fluorescence images and a pseudocolored ratio image. The average $F_{\text{green}}/F_{\text{red}}$ was 0.274 ± 0.006, corresponding to an ASL [K⁺] of 16.0 ± 0.7 mM (S.E., 7 mice) (Fig. 9D). Thus, as found for the human airway epithelial cell cultures, ASL [K⁺] in intact pig and mouse tracheas was substantially greater than that of 5 mM in the bathing medium or serum.

**DISCUSSION**

We previously reported on the values and determinants of [Na⁺], [Cl⁻], pH, and osmolality in the ASL (7, 9). Here, utilizing a new, long wavelength, ratioable [K⁺]-sensing fluorescent indicator, we report the characterization of ASL [K⁺]. We found that [K⁺] in the ASL in airway epithelial cell cultures and intact trachea, 17–25 mM, is well above that of the basolateral membrane bathing solution (or serum) of ~5 mM. Passive Donnan equilibrium cannot account for this 3–5-fold increase in [K⁺], because transepithelial PD in the airway is only 4–30 mV (29), and transepithelial PD of the human airway cell cultures in this study was ~7 mV. Therefore, active transcellular transport must be involved, which was supported experimentally by the reduced ASL [K⁺] following ouabain and the relative insensitivity of ASL [K⁺] to large changes in perfusate [K⁺]. A high ASL [K⁺] has been proposed to facilitate ASL acidification by the apical H⁺/K⁺ pump (27); we confirmed expression of mRNA encoding the apical H⁺/K⁺ pump ATP1AL1 in both primary cultured and freshly isolated airway epithelial cells. In contrast to the high ASL [K⁺], ASL [Na⁺], [Cl⁻], osmolality, and pH were found previously to be similar to those of the basolateral membrane bathing solution (7, 9).

The effects of transport modulators provide clues about the transporters involved in maintaining a relatively high ASL [K⁺]. Ouabain predictably reduced ASL [K⁺] toward that of the perfusate, because the basolateral Na⁺/K⁺ pump is the principle active transporter responsible for establishing electrochemical gradients. Under open circuit conditions, the basolateral Na⁺/K⁺ pump produces a positive transcellular current from apical-to-basolateral sides, resulting in a small, apical-negative transepithelial PD. The reduced ASL [K⁺] following amiloride, CFTRinh-172, TEA, and XE991 suggests the involvement of ENaC, CFTR, and K⁺ channels in maintaining the high ASL [K⁺]. The electrochemical gradient favors apical cellular K⁺ efflux through K⁺ channels, which may be facilitated by intrinsic outward rectification as reported for some apical membrane epithelial K⁺ channels (12, 13). The ENaC and CFTR inhibition effects are indirect, acting in part by apical membrane hyperpolarization and reduced transepithelial PD. A full quantitative description of the determinants of the high ASL [K⁺] will require mathematical modeling, as we did previously for airway (30) and corneal (31) epithelia bathed in infinite, well stirred solutions, as well as measurements of intracellular concentrations of each of the ions and apical and basolateral membrane potentials.

Our results thus indicate the involvement of multiple K⁺ channels in anion secretion and regulation of ASL [K⁺] in human airway epithelia, and extend prior data on the identification of K⁺ channels expressed in airway epithelia. Calu-3 cells express mRNA encoding KCNQ1, KCNQ2, and KCNE3, which are involved in cAMP-activated anion secretion, and KCNN4, which contributes to Ca²⁺-activated anion secretion (24). As shown in Fig. 6B, KCNQ channel inhibition blocked forskolin and Ca²⁺-stimulated $I_{\text{sc}}$ in HBE cells, suggesting an important role for KCNQ channels and their accessory KCNE subunit in cAMP and Ca²⁺-stimulated anion secretion. We found expression of mRNA encoding KCNQ1, KCNQ3, KCNQ5, KCNE1–4, and KCNN3–4 in both primary cultured and freshly isolated HBE cells (Fig. 7C). By immunofluorescence (Fig. 7D), we found apical and basolateral expression of KCNQ1 and apical localization of KCNQ3 and KCNQ5. The effects of chromanol 293B and
XE991 support the functional expression of KCNQ1, KCNQ3, and KCNQ5 in HBE cells. Together, these results indicate the involvement of KCNQ channels in the regulation of ASL [K⁺].

The similar ASL [K⁺] in non-CF and CF airway epithelia suggests that dysregulation of ASL [K⁺] is not involved in the pathophysiology of CF lung disease, as was concluded previously for ASL [Na⁺] and [Cl⁻]. The small difference in ASL [K⁺] in CF airway epithelia and CFTRinh-172-treated non-CF epithelia may be related to different compensatory mechanisms and/or transporter expression in CF cultures, which chronically lack CFTR function, versus non-CF cultures that are acutely treated with CFTRinh-172.

The K⁺ sensitivity of the ratioable fluorescent K⁺ indicator used here is based on K⁺-selective binding to the triaza- cryptand chromophore, where one K⁺ ion binds tightly within the two ethylene glycol bridges, which coordinate to the K⁺ ion through electrostatic interactions involving the electronegative oxygen and nitrogen atoms contained within the triazacryptand ring. With increasing [K⁺], K⁺-triazacryptand binding blocks charge transfer fluorescence quenching of the Bodipy chromophore in TAC-Lime, resulting in increased Bodipy fluorescence. Here, we designed and synthesized a dual wavelength K⁺ indicator by dextran conjugation of TAC-Lime and TMR. The fluorescence properties and K⁺ sensitivity of TAC-Lime were not affected by the dextran conjugation. The dextran backbone conferred high water solubility and membrane impermeability, allowing staining of the aqueous ASL without significant cellular uptake. The K⁺-sensing dextran conjugates should have wide applications to K⁺ measurements in extracellular fluid compartments such as brain and intracellular organelles accessible by fluid phase endocytosis.

Measurement of ASL [K⁺] involved ratio imaging after TLT-dex staining of the ASL. Dye staining/imaging offers significant advantages over existing methods to measure ASL [K⁺] that require sampling or direct contact with ASL fluid. Surface tension forces associated with filter paper and microcapillary sampling methods draw excess fluid from cells and underlying structures, confounding the interpretation of [K⁺] in the sampled fluid. K⁺-sensitive double-barrel microelectrodes are used by few laboratories because they require considerable technical expertise. Also, K⁺-sensitive microelectrodes require direct contact with ASL fluid and allow measurement only at the contact site where the ASL is perturbed. Fluorescent indicators and imaging methodology allow precise spatial and temporal mapping of [K⁺] without contacting the ASL.

The relatively high [K⁺] measured in the ASL of intact pig and mouse trachea confirmed the major conclusion from measurements on airway epithelial cell cultures. Previous estimates of ASL [K⁺] in tracheas of pig, rat, and mouse by x-ray microanalysis were ~20, 13, and 28 mM, respectively (32). ASL [K⁺] measurement by flame photometry following ASL sampling of human trachea and bronchi by filter paper gave ASL [K⁺] of ~22 mM (5). Notwithstanding the caveats using these methods as mentioned in the Introduction, our results here are in agreement with the prior data.

In summary, we developed a minimally invasive fluorescence method to measure [K⁺] in the ASL. Measurements using various transporter modulators indicated the principal determinants in maintaining a relatively high ASL [K⁺]. Together with existing fluorescent indicator methods to measure extracellular [Na⁺], [Cl⁻], osmolality, and pH, it is now possible to image each of the major ions in extracellular space fluids.

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