A ‘Virtual Gland’ method for quantifying epithelial fluid secretion

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ABSTRACT

We developed a new apparatus, the virtual gland (VG), for measuring the rate of fluid secretion ($J_v$), its composition, and the transepithelial potential (TEP) in cultured epithelial cells under open circuit. The VG creates a 10 $\mu$l chamber above the apical surface of epithelial cells on a Costar filter with a small hole leading to an oil-filled reservoir. After priming with a fluid of choice, secreted fluid is forced through the hole into the oil where it forms a bubble that is monitored optically to determine $J_v$ and collected for analysis. Calu-3 cells were mounted in the VG with a basolateral bath consisting of Krebs-HEPES buffer at 37°C. Basal $J_v$ was 2.7 ± 0.1 $\mu$l/cm$^2$/hr ($n=42$), and TEP was $-9.2 \pm 0.6$ mV ($n=33$); both measures were reduced to zero by ouabain ($n=6$). $J_v$/TEP were stimulated 64/59% by 5 $\mu$M forskolin ($n=10$), 173/101% by 1 mM 1-EBIO ($n=5$), 213/122% by 333 nM thapsigargin ($n=5$), and 520/240% by combined forskolin and thapsigargin ($n=6$). Basal $J_v$/TEP were inhibited to 82/63% with 10 $\mu$M bumetanide ($n=5$), 71/82% with 100 $\mu$M acetazolamide ($n=5$), and 47/56% with 600 $\mu$M glibenclamide ($n=4$). Basal $J_v$/TEP were 52/89% of control values after HCO$_3^-$ replacement with HEPES ($n=16$). The net [HCO$_3^-$] of the secreted fluid was close to bath (25 mM) except when stimulated with forskolin or VIP, when it increased (~80 mM). These results validate the use of the VG apparatus and provide the first direct measures of $J_v$ in Calu-3 cells.

Keywords: cystic fibrosis, submucosal gland, serous cell, mucus, airway, secretion
INTRODUCTION

People with the genetic disease cystic fibrosis (CF) typically die from unremitting lung infections. Susceptibility to these infections is generally considered to arise from dysfunction of electrolyte and fluid transport across airway epithelia. Transport dysfunction is caused by loss of function of CFTR, an anion channel found primarily in the apical membranes of epithelial cells. Both ciliated surface epithelia and submucosal gland serous cells express CFTR, and both of these cell types presumably contribute to the etiology of CF airway disease, but glands are the major secretory organs of healthy lungs. When provoked, they produce copious amounts of antibiotic-rich mucus that traps pathogens and inhibits their growth while they are swept from the airways (28). Mucus clearance normally keeps the airways sterile, and it has been proposed that altered gland function in CF airways impairs the mucus shield (1, 15-17, 21, 23, 26, 49, 52, 54).

Airway glands can be studied in isolation and a great deal has been learned about their regulation (42). Gland mucus secretion has been quantified extensively (1, 6, 15-17, 23, 25, 26, 38, 47-50), but because glands are difficult to study with electrophysiological methods, electrophysiological study of cultured monolayers of gland cells and cell lines has mainly been used (31, 55-58). However, except in rare cases (22, 45) fluid transport by cell sheets has not been quantified. Therefore, we sought a simple method that would help bridge the gap between studies of native glands and cultured cell sheets.

We have now developed a novel method for measuring fluid secretion across epithelial cell sheets, and have used it to quantify the amount and selected features of fluid secretion by Calu-3 cells, a widely studied surrogate for airway submucosal gland serous cells (39). Our method differs in several respects from prior studies of Jv in airway epithelia (22, 45). In the best of these, Miller et al. used an elegant modification of the capacitance probe technique (51) to measure fluid absorption across the retinal pigment epithelium with an accuracy of 0.5-1.0 nl/min (34), and subsequently their method was used to clarify important points of transport in several systems (5, 10, 37), including gland cells from normal and CF subjects (22). In the capacitance probe apparatus the epithelial cell sheet separates two large volumes (~12 ml) of fluid. The chambers are sealed except for a single thin column of fluid that moves relative to a probe that detects the capacitance between it and the surface of the fluid. The advantages of the capacitance probe are its sensitivity and ability to measure fluid flow in either direction. A
disadvantage is that the large volumes of fluid used essentially clamp the fluid composition, so that, unlike the natural situation, the cells do not determine the composition of their apical fluid. The large volumes also mean the composition of the secreted fluid is unknown.

The apparatus introduced here, which we term a ‘Virtual Gland’, is advantageous for studies of secretory epithelia because it allows the formation and collection of the secreted fluid. The virtual gland has about the same sensitivity as the capacitance probe, is simpler to use, and because the bath is an open system, secretion rates are not altered by variations in the height, volume, or temperature-related volume changes of the bath. In addition to being able to measure the composition of the secreted fluid, this system has the additional advantage that the cells can respond to that fluid, as we expect they do in real glands. The ionic concentration of the secreted fluid will help set the apical transmembrane potential, and secreted mediators, for example ATP (8), can act back on the cells to influence the secretory process.

In the present studies we introduce the VG method and use it to provide direct evidence that Calu-3 cells secrete fluid in open circuit conditions. In Ussing chamber experiments they produce a basal short circuit current ($I_{sc}$) of 20-40 $\mu$A/cm$^2$ that is mainly the result of $\text{HCO}_3^-$ secretion. Stimulation with forskolin causes a further increase in $\text{HCO}_3^-$ secretion, while stimulation with agents that hyperpolarize the cell, such as thapsigargin and 1-EBIO, recruit mainly Cl$^-$-mediated increases in $I_{sc}$ (4, 31, 36, 39).

We determined the magnitude and properties of Calu-3 fluid secretion in response to various mediators and inhibitors, and compared the results with predictions made from $I_{sc}$ studies (4, 11-14, 18, 19, 27, 30-32, 35, 36, 39, 40, 43, 44, 53), and with secretion by native airway submucosal glands (21, 23-26, 54).
METHODS

**Cell culture**—The Calu-3 cell line was obtained frozen from the American Type Culture Collection (Rockville, MD). After thawing, the cells were grown at 37°C in T25 tissue culture flasks (Costar, Pleasanton, CA) containing 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12, plus 15% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine in an atmosphere of 5% CO₂ - 95% O₂. Cell were passaged with 1:8 dilution and plated at 5 x 10⁵ cell/cm² onto Costar Snapwell filters (12 mm diameter, 0.4 µm pore size, 1 cm² growing area, Costar, Cambridge, MA) that had been coated with human placental collagen (Sigma). Cells were grown at the air/liquid interface for at least 14 days before use. In the present study, the mean age of the cells was 21 ± 1 days (n=70).

**Virtual gland apparatus**—The prototype virtual gland (VG) is shown in Fig. 1. A Costar Snapwell filter containing confluent cells is assembled as shown so that a 1.7 mm thick plastic barrier creates a small volume *apical chamber* above the apical surface of the cells. The barrier separates a thin film (~90 µm depth, ~10 µl) of apical fluid from a water-saturated mineral oil layer (500 µl). A 0.6 mm diameter hole in the barrier serves as a *virtual duct* to convey the apical fluid into the oil-filled *collection chamber*, where it forms a spherical bubble whose diameter can be measured optically at regular intervals and converted to a volume; the change in volume over time provides a direct measure of Jᵥ (see below). A second smaller hole serves as a *voltage port* (see below).

Before assembly the apical surface of the cells is rinsed 2X with Krebs-Ringer bicarbonate buffer (KRB) without glucose and then 2X with the same solution containing 10 µM Blue Dextran. The filter cup and the collection chamber are then both filled with the blue dextran solution and the collection chamber is pressed gently but firmly into the Snapwell filter cup while the filter is supported against a flat, KRB-covered surface to prevent it from being distended by the pressure of assembly. Excess apical fluid is expressed through the hole into the collection chamber, and the purpose of having fluid of the same composition in that chamber at this stage is to prevent the formation of bubbles in the apical chamber which can otherwise occur during assembly. Because of variations in dimensions of the filters, cell layers and assembly
procedures, assembled VGs have apical chambers of variable volumes, which thus must be measured in each experiment.

To estimate the volume of the apical chamber, the bubble of apical fluid is collected at intervals during secretion and the blue dextran concentration [BD] determined colorimetrically. The apical chamber volume is then given by:

\[
V_A = V_{SF} \times \frac{[BD]_{SF}}{([BD]_{initial} - [BD]_{SF})}
\]

Where:

\[
\begin{align*}
V_A &= \text{volume of apical chamber} \\
V_{SF} &= \text{volume of secreted fluid} \\
[BD]_{SF} &= \text{blue dextran concentration in the collected fluid bubble} \\
[BD]_{initial} &= \text{starting concentration of blue dextran (initially 10 µM, and then reset after each measurement).}
\end{align*}
\]

In the present study, the estimated mean volume of the apical chamber was 10.0 ± 1.0 µl (n=60).

The assembled Snapwell filter and collection chamber is placed in a fabricated plastic bath holder so that the cell layer is positioned just at the surface of the bath KRB solution (volume ~5 ml) to eliminate any significant positive or negative hydrostatic pressure. The bath KRB was continuously gassed with 5% CO₂-95% O₂ and maintained at 37°C with a TS-4 temperature controller (Sensortek, Inc.) that was coupled to the bath chamber by an aluminum jacket machined to fit the chamber. The KRB composition was: 115 mM NaCl, 2.4 mM K₂HPO₄, 0.4 mM KH₂PO₄, 25 mM NaHCO₃, 1.2 mM CaCl₂, 2 mM glucose. pH was 7.4, and osmolarity was adjusted to 290~295mOsm. For HCO₃⁻-free experiments, all HCO₃⁻ in the KRB was replaced either with 25 mM HEPES or 1 mM HEPES plus 24 mM NaCl that had been pre-gassed with humidified 100% O₂. The HEPES solutions were adjusted to pH at 7.4 after gassing with O₂ and all HEPES experiments were performed with continuous O₂ gassing.

Measuring \(J_v\) and collecting the secreted fluid—Dynamic measurements of \(J_v\) are obtained by optical monitoring of the growth of the fluid bubble in the oil (Fig 1e, 1f, and 2). We used a Wild microscope fitted with a digital video camera (Logitech, Fremont, CA) and captured images automatically at fixed intervals (typically 5 min) using software supplied with the camera. An optical grid (0.5 mm, Fig. 1e) was present throughout for calibration. The diameter or areas of
digital images of bubbles were measured with a commercial version of NIH Image software (Scion, Frederick, MD) and the measures converted the volume using the formula for a sphere. J_v was determined for each 5 min period as \([\text{volume of bubble n+1} - \text{volume of bubble n}] \div \text{time}\). For presentation of ‘continuous’ data (e.g. Fig. 5), two 5 min periods were averaged. For summary data in response to agonists or inhibitors, a 20 min period of secretion starting with the peak response was used. In rare cases when the response continued to increase (agonists) or decrease (inhibitors) until the end of the 30-40 min epoch, the last 20 min of the epoch was used.

All measured bubbles were subsequently collected between oil blocks in constant bore capillary tubing (usually 10 µl volume, Drummond Scientific Co., Broomall, PA) and the linear dimension of the collected fluid column again measured at 60X using a Wild binocular microscope. The volume is then given by the formula for a cylinder or merely as a fraction of the preset total tube volume. The two measurement procedures showed good agreement for bubble volumes under 3.0 µl; the mean collected volume was 96.4 ± 0.4% of the digitally calculated volume (195 bubbles, 60 experiments). As bubbles increase in size the spherical shape becomes oblate and the assumption of spherical shape leads the volume to be overestimated. The shapes of the bubbles were directly observed during calibration procedures by imaging them from both the top and from the side via prism (Melles Griot, Rochester, NY). The volumes used for all calculations of J_v were taken from bubbles under 3 µl and were checked and if necessary corrected from measurements in constant bore capillaries. Collected fluid was stored frozen for subsequent physical and biochemical analyses.

**Measurement of Transepithelial Potential Difference**—TEP was measured via a *voltage port* consisting of a 0.36 mm diameter hole filled with a wick of conducting material (we used a buffer-saturated fragment of absorbent paper) that established electrical continuity with the apical fluid and allowed the TEP to be measured with a microelectrode while preventing bulk flow of fluid (Fig. 1e). The microelectrode was pulled with a vertical puller and the tip broken to give low impedance when filled with bath solution. It was coupled via silver-silver/chloride wire to a high impedance microelectrode amplifier (Getting Instruments). During the course of these experiments we discovered that Calu-3 cells are very sensitive to silver chloride, showing a rapid drop in TEP and J_v in response to a ground wire in the small volume bath (for similar findings with smooth muscle confer ref (20)). Therefore, the circuit was completed and the bath grounded...
via a 4% agar bridge made up in bath solution. The TEP was monitored and recorded continuously on a PC using Power Lab 4/20 interface and software (ADInstruments Pty Ltd, Castle Hill, Australia, Fig. 1g). A comparison of TEP measured at the voltage of port and in the actual fluid bubble showed that they were virtually identical.

Measurement of pH—Collected fluid was placed in micro chambers designed to accommodate the tip of a mini-combination pH electrode (WPI, Inc., Sarasota, FL), and pH measured under conditions of constant temperature and nominally 100% humidity (no evaporation was detected during the course of the measurements). Because the apical chamber must be primed with an added fluid, early collections are mixtures of the secreted and the priming fluid and appropriate corrections must be applied. We start by knowing the volume of the apical chamber and its starting pH, we then measure the volume of secreted fluid and the pH of the collected fluid. The pH values are converted to $\text{HCO}_3^-$ concentrations and then to nanomoles of $\text{HCO}_3^-$ in the apical and collection chamber (i.e. the fluid bubble). The increase in nanomoles of bicarbonate divided by the secreted volume (collection chamber volume only) is the average concentration of bicarbonate secreted over the sample interval.

Reagents—Forskolin, thapsigargin, acetazolamide, bumetanide and ouabain were obtained from Sigma. 1-Ethyl-2-benzimidazolinone (1-EBIO) was purchased from Aldrich Chem. Co. (Milwaukee, WI). Ouabain was dissolved in deionized water at a stock concentration of 10 mM. A 10 mM stock solution of bumetanide was made by dissolving in 200 mM sodium hydroxide. All other drugs were dissolved in dimethylsulfoxide (DMSO) at the following concentrations: forskolin, 10 mM or 50 mM, thapsigargin, 1 mM, acetazolamide, 200 mM, 1-EBIO, 2 M and glibenclamide, 300 mM. In control experiments we observed that DMSO levels >0.15%, depressed basal $J_v$, so we kept the final concentration of DMSO at or below 0.15% where possible.

Statistics—All data are expressed as means ± SEM, and $n$ indicates the number of experiments. Statistical difference was determined by Student’s $t$ test. A value of $p<0.05$ was considered statistically significant.
RESULTS

Basal secretion—Filters mounted in the virtual gland showed high and variable rates of fluid secretion immediately after mounting, but declined to relatively stable values within 30-60 min. Therefore, we ignored the first 60 min of data and followed basal secretion for up to 6 additional hours. Summary data for four long-term basal secretion experiments is shown in Fig. 3, and mean basal secretion rates for a much larger sample taken 60-90 min post-mounting are shown in Fig. 5. For all data the average basal secretion rate was $2.7 \pm 0.1 \mu l/cm^2/hr$ (n=42) and the average basal TEP was $-9.2 \pm 0.6 mV$, (n=33).

Several control experiments were carried out to verify that the observed fluid accumulation represents active secretion. When filters without cells or filters with killed cells were placed in the VG apparatus, no fluid accumulation was observed (data not shown). Ouabain blocks Na$^+$, K$^+$, ATPase and eliminates all $I_{sc}$ in Ussing chamber experiments with Calu-3 cells (44). When applied to Calu-3 cells in the VG, ouabain (10 $\mu M$) caused a slow decline in TEP (time constant $= 28 \pm 1$ min, n=6) and the $I_v$ (time constant $= 39 \pm 3$ min, n=6). After 90 minutes both values reached zero.

Responses to agonists—An abundant literature describes $I_{sc}$ responses of Calu-3 cells to forskolin, thapsigargin, and 1-EBIO. Forskolin elevates $[cAMP]_i$ and produces variable increases in $I_{sc}$ that are inversely proportional to the level of basal $I_{sc}$. Thapsigargin elevates $[Ca^{2+}]_i$, by inhibiting the sarcoplasmic reticulum Ca$^{2+}$-ATPase (46), and 1-EBIO activates both CFTR and basolateral K$^+$ channels (2, 3). All of these agonists stimulated increases in fluid secretion from Calu-3 cells in the Virtual Gland.

**Forskolin** (5 $\mu M$) increased $J_v$ from $2.4 \pm 0.3$ to $4.0 \pm 0.4 \mu l/cm^2/hr$ ($64 \pm 16 \%$, n=10) and increased TEP from $-7.6 \pm 0.8$ to $-12.1 \pm 1.2 $ mV ($59 \pm 16\%$, n = 10, (Fig. 4). VIP (1 $\mu M$) increased $J_v$ from $3.1 \pm 0.4$ to $5.4 \pm 0.8 \mu l/cm^2/hr$ ($71 \pm 25\%$, n=4, data not shown). **Thapsigargin** (333 nM) increased $J_v$ from $2.0 \pm 0.2$ to $6.4 \pm 0.9 \mu l/cm^2/hr$ ($213 \pm 44 \%$, n=5) and increased the average TEP from $-8.2 \pm 1.2$ to $-18.2 \pm 3.3 $ mV ($122 \pm 40\%$, n=5, Fig. 4, 5). In some experiments we noted oscillations in the TEP as has been reported for the $I_{sc}$ after stimulation with thapsigargin (Fig. 1g), but the sampling limits for determining $J_v$ did not allow us to determine if it showed similar oscillations. When forskolin and thapsigargin were used in
combination, the response was synergistic, increasing the \( J_v \) from 2.0 ± 0.5 to 12.2 ± 1.0 µl/cm²/hr (520 ± 50 % of basal \( J_v \)) and TEP from −8.2 ± 1.1 to −28.0 ± 2.2 mV, (240 ± 30 %, n=6, Fig. 4, 5). **1-EBIO** stimulates large \( I_{sc} \) increases from Calu-3 cells in Ussing chamber experiments. For Calu-3 cells mounted in the virtual gland, 1-EBIO (1 mM) increased fluid secretion from 3.2 ± 0.4 to 8.6 ± 1.0 µl/cm²/hr (173 ± 33 %, n=5) and TEP from −10.1 ± 1.1 to −20.3 ± 3.5 mV (101 ± 34 %, n=5, Fig. 4).

**Responses to inhibitors**—Effects of 5 inhibitory conditions on basal \( J_v \) and TEP are summarized in Fig. 6. Interactions between inhibitors and agonists on \( J_v \) are presented below.

**Bumetanide** inhibits the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC), which is responsible for transporting Cl⁻ into the cell across the basolateral membrane. To estimate the contribution of NKCC-dependent Cl⁻ secretion to \( J_v \) and TEP, we tested the effects of bumetanide (10 µM) on basal and stimulated secretion. Bumetanide reduced basal \( J_v \) from 2.5 ± 0.3 to 2.1 ± 0.3 µl/cm²/hr (18 ± 11 %) and TEP and from -9.2 ± 0.6 to -5.8 ± 1.1 mV (37 ± 11 %, n=5, Fig. 6). The response to forskolin was not affected by bumetanide, but the response to thapsigargin was eliminated: addition of thapsigargin after bumetanide failed to increase either \( J_v \) 2.1 ± 0.3 to 2.2 ± 0.5 µl/cm²/hr (3 ± 22 %) or TEP -5.8 ± 1.1 to -6.9 ± 1.7 mV, (20 ± 30 % (n = 5, data not shown). When bumetanide was applied following thapsigargin stimulation it inhibited almost 100% of the stimulated \( J_v \) and TEP (n=5, Fig. 5). When bumetanide followed 1-EBIO stimulation, it had differential effects on \( J_v \) and TEP. Bumetanide inhibited most but not all of the \( \Delta J_v \), leaving \( J_v \) ~ 35% higher than basal values. In contrast, the TEP was inhibited to a level 65% below the basal value (n=5, data not shown). We have no explanation for this unusual effect. Because of the large response to forskolin + thapsigargin, we were interested to see the effect of bumetanide on these agents used in combination. Bumetanide reduced the \( J_v \) from 11.6 ± 1.1 to 6.8 ± 0.7 µl/cm²/hr (-42 ± 6%, n=5) and the TEP from 21.3 ± 4.1 to 9.7 ± 1.7 mV (-55 ± 8%, n=5). We noted that the \( J_v \) remaining after bumetanide was significantly (p < 0.001) greater than the basal value of 2.0 ± 0.5 µl/cm²/hr, suggesting that, in the presence of forskolin, thapsigargin can slightly increase the secretion of a bumetanide-insensitive component, which is most likely HCO₃⁻.

**Acetazolamide**, which inhibits carbonic anhydrase, was previously shown to reduce the basal \( I_{sc} \) of Calu-3 cells by ~ 1/3 (44). In the virtual gland, acetazolamide (100 µM) reduced
basal $J_v$ from $2.3 \pm 0.2$ to $1.6 \pm 0.3$ $\mu l/cm^2/hr$ ($29 \pm 11\%$) and TEP from $-8.2 \pm 1.1$ to $-6.7 \pm 1.1$ mV ($18 \pm 14\%$, n=5, Fig. 6). Stimulation by forskolin ($5 \mu M$) was blocked after acetazolamide: neither $J_v$ nor TEP showed a significant increased $\Delta J_v = 10 \pm 20\%$ and $\Delta TEP = 10 \pm 10\%$ (n = 5, n.s. data not shown).

**HEPES (HCO$_3^-$-free condition).** For these experiments, filters were mounted in HCO$_3^-$-free medium (both bath and apical fluid, with either 1 or 25 mM HEPES addition to maintain pH), and were gassed with O$_2$. In this condition we measured basal secretion from 30-60 min post-mounting. The average basal $J_v$ in HCO$_3^-$-free medium (both bath and apical fluid) was $1.4 \pm 0.2 \mu l/cm^2/hr$ (n=16) vs. $2.7 \pm 0.1 \mu l/cm^2/hr$ in Krebs, (n=42), a reduction of 50% ($p < 0.005$), but the TEP was reduced by only 12% ($-8.1 \pm 0.8$ (n=16) vs. $-9.2 \pm 0.6$ mV) (n=33, n.s.), Fig. 6. In the absence of HCO$_3^-$, forskolin was without effect and did not alter the gradual decline in $J_v$ or TEP (n=8); this is agreement with observations of $I_{sc}$ in Ussing chambers (4). The subsequent addition of thapsigargin caused large increases in $J_v$ ($0.7 \pm 0.2$ to $5.1 \pm 1.7 \mu l/cm^2/hr$, $598 \pm 228\%$, n=6), and TEP ($-5.4 \pm 1.5$ to $-21.9 \pm 5.3$ mV, $303 \pm 97\%$, n=6), but these increases were transient and were followed by rapid declines back to baseline values Fig. 7).

**Bumetanide plus HCO$_3^-$-free conditions.** Addition of bumetanide to Calu-3 cells mounted in zero HCO$_3^-$ reduced the basal secretion rate from $1.2 \pm 0.3$ to zero $\mu l/cm^2/hr$ within 30 min (n=4), but the TEP was only reduced from $-7.7 \pm 0.9$ to $-4.8 \pm 1.1$ mV ($37 \pm 14\%$, n=5, Fig. 6 and Fig. 8). Additional ouabain reduced the TEP to zero mV within 60 additional min (data not shown).

**Glibenclamide** is a non-specific inhibitor of CFTR (41). Addition of 600 $\mu M$ glibenclamide basolaterally reduced the basal $J_v$ and TEP by 49 and 46%, to $1.8 \pm 0.3 \mu l/cm^2/hr$ and $-6.8 \pm 1.9$ mV (n=4). Addition of forskolin after glibenclamide produced no increase in either $J_v$ or TEP (n=3), but subsequent addition of thapsigargin caused a 98% increase in $J_v$ to $2.7 \pm 0.5 \mu l/cm^2/hr$ (n=4). However, this occurred in the presence of a decrease in TEP (mean change of $-21\%$ to $-4.5 \pm 0.5$ mV). The $J_v$ changes to thapsigargin + forskolin were highly variable in the presence of glibenclamide, ranging from zero to 284%, and the maximum $J_v$ observed was only $3.6 \mu l/cm^2/hr$, vs. a mean $J_v$ of $\sim 12 \mu l/cm^2/hr$ observed in the absence of glibenclamide. In 3 experiments glibenclamide was added after forskolin and the mean result was to reduce $J_v$ back to basal levels. Note that for maximum effectiveness glibenclamide is usually added bilaterally, but was only added basolaterally in these experiments.
Relationship between mean $J_v$ and mean TEP in the Virtual Gland.—The transepithelial potential difference ranged from zero to -38 mV, and showed an approximately monotonic relation to $J_v$, with each 2.8 mV of TEP equating to a $J_v$ of ~ 1 µl/cm²/hr (Fig. 9). If secretion is purely isotonic (150 mM) 1 µl/cm²/hr should correspond to ~4 µA, which would give a mean resistance for the filters of 700 $\Omega \cdot$ cm².

The peak $J_v$ in response to every agonist was always delayed relative to the peak TEP and decreases of TEP for every inhibitor also always preceded decrease in $J_v$, indicating some physical capacitance in the system. Inspection of many records indicated a mean delay interval of ~ 10 min between TEP and $J_v$, and so the above correlations were always made with that time correction. Taking all measures together, it appeared that fluid secretion by Calu-3 cells always required an electrogenic component, however little it might be. On the other hand, as the data of Fig. 8 show, it was possible to maintain a TEP in the absence of active secretion. An interesting point is that we never saw fluid absorption with Calu-3 cells, although absorption was readily apparent with other cell types (Tirouvanziam et al. ms in preparation), and could be induced in Calu-3 cells by making the bath hypertonic.

Comparison of Calu-3 responses in VG with Calu-3 $I_{sc}$ responses and native gland secretion
—A comparison of responses to agonists for Calu-3 cells in the VG vs. in the Ussing chamber is shown in Table 1 which also includes the response for these same agonists observed in native glands. Note that agonists and antagonists can only be added basolaterally in the VG and for real glands. The $J_v$ and $I_{sc}$ responses were qualitatively similar, while the main difference in comparison to glands is that thapsigargin had little effect on native glands, while carbachol has little effect on Calu-3 cells. If Calu-3 cell responses to thapsigargin are compared to gland responses to carbachol, it is seen that responses to carbachol and forskolin are completely occlusive in glands, whereas they are additive and possibly synergistic in Calu-3 cells. We hypothesize that activation of muscarinic receptors in glands can't be mimicked merely by elevating [Ca²⁺].

$[HCO_3^{-}]$ in secreted fluid.—An advantage of the virtual gland system over prior methods is that the secreted fluid can be collected and assayed for any desired property. However, because the
apical chamber must be primed with an added fluid, early collections are mixtures of the secreted and the priming fluid and appropriate corrections must be applied (see methods). We measured the pH of collected fluid with a miniature pH electrode and converted the values to HCO$_3^-$ concentrations by the Henderson-Hasselbalch equation (Fig. 10). With a bath pH of 7.4 (25 mM HCO$_3^-$), the mean HCO$_3^-$ concentration of basal secretions was 36 ± 9 mM (n=12). Forskolin or VIP stimulation increased [HCO$_3^-$] (to 74 ± 21 mM, n=5, and 92 ± 39 mM, n=4 respectively), while thapsigargin stimulation decreased [HCO$_3^-$] to 17 ± 4 mM, (n=6), and thapsigargin + forskolin produced a [HCO$_3^-$] of 23 ± 4 mM (n=4). According to these data, the secreted fluid was similar to bath pH except when stimulated with forskolin or VIP, when it became markedly more basic.

The levels of basal secretion observed in the VG varied considerably. For the experiments reported in Fig. 4, mean basal $J_V$ was slightly above 2 µl/cm$^2$/hr, and direct, continuous measures of pH taken with pH-sensitive microelectrodes during secretion indicated that the basal secretions were more acidic than the bath (data not shown). In contrast, pH of the collected basal secretions from earlier experiments indicated a more basic secretion. When the two sets of data were compared, it became apparent that basal $J_V$ in the earlier experiments was much higher than in recent experiments. Therefore, we compared the calculated [HCO$_3^-$] of basally secreted fluid as a function of $J_V$. As shown in Fig. 11, the function was positive.
DISCUSSION

In this paper we introduced a novel method to study fluid secretion by epithelial cell sheets in open circuit conditions, and applied it to fluid secretion by the Calu-3 cell line.

Overview of the virtual gland (VG) method.—The VG (i) provides a direct measure of fluid secretion, (ii) operates in open circuit conditions, (iii) allows the cells to form their own apical fluid, (iv) allows them to respond to the composition of that fluid, (v) allows the fluid to be collected for analysis, (vi) and allows the transepithelial potential difference to be measured.

Open circuit studies are important adjuncts to I_sc studies because ion flows can differ markedly in the two circumstances (10). In addition, the VG method can in principle identify electrically silent mechanisms of fluid transport that are missed in I_sc studies. Because the apical fluid compartment seen by the cells has a relatively small volume the cells can create a distinctive apical fluid similar to what they would experience in vivo. That can't happen in the Ussing chamber or capacitance probe methods, where the fluid composition is clamped by the large volumes present. Because the apical fluid in the VG can approximate the composition of native fluid, the cellular ion transport mechanisms may be influenced in a similar manner by the altered ion gradients that develop, as well as by secreted mediators such as ATP (8, 9) and the myriad proteins released by Calu-3 cells (33, 59). For all of these reasons the VG method can provide an essential link between Ussing chamber experiments and studies of fluid transport by intact glands.

Limitations of the virtual gland (VG) method.—(i) In its present form the VG doesn't measure epithelial resistance, which weakens inferences about possible contributions of electrically silent transport mechanisms to fluid secretion. (ii) The VG would also be improved by reducing the volume of the apical chamber from 10 to 2 µl, which would correspond to a chamber height of ~18 µm. That was achieved in some experiments, but at increased risk of cell damage. (iii) The apical chamber is relatively inaccessible, so in this study all reagents were added only to the basolateral side. (iv) The secretion of fluid as a bubble into an oil layer eliminates evaporation and makes possible a sensitive optical assay of J_v, but lipophilic components of the fluid may be lost. (v) Finally, the present system requires the development of skills for assembly of the VG
and for collection of the secreted fluid. All of these limitations can be addressed with future development of the system. With these advantages and disadvantages in mind, what have we learned about Calu-3 cells from this initial VG study?

**Basal secretion by Calu-3 cells in the VG**—Calu-3 cells are widely used as models for airway gland serous cells, and are interesting because anion transport in the Ussing chamber is either HCO$_3^-$-rich or Cl$^-$-rich depending upon the mode of stimulation (4). In Ussing chamber experiments, Calu-3 cells show varying levels of basal $I_{sc}$, which is characterized by relative insensitivity to bumetanide (36). Calu-3 cells in the VG always displayed a resting $J_v$ that averaged 2.7 $\mu l/cm^2/hr$. The $J_v$ varied greatly among different filters (see **Fig. 11**), but for any given filter the $J_v$ was stable for many hours after an initial brief period of higher secretion that probably was induced by aspects of the mounting procedure. Bumetanide reduced basal $J_v$ by only 18%, whereas the replacement of HCO$_3^-$ with HEPES buffer reduced basal $J_v$ by 52%, and the subsequent addition of bumetanide reduced $J_v$ to zero. We interpret these results to mean that basal $J_v$ is mediated by both Cl$^-$ and HCO$_3^-$ secretion, and when transport of one anion is inhibited the other can partially compensate. The [HCO$_3^-$] of basally secreted fluid was positively related to $J_v$, and at lower $J_v$ could actually be less than the bath concentration. This seems inconsistent with other evidence for high levels of HCO$_3^-$ secretion, and we now have evidence from pH-stat experiments that acid secretion is also occurring (29).

**Forskolin-stimulated $J_v$**—Forskolin was used as surrogate for the natural transmitter VIP (23), which was only used in a few experiments. Forskolin increased $J_v$ to 4.0 $\mu l/cm^2/hr$, and this increase was completely eliminated by prior treatment with acetazolamide or by replacement of HCO$_3^-$ with HEPES, indicating that the stimulation of $J_v$ by forskolin was entirely mediated by increased HCO$_3^-$ transport (4) and that, unlike basal $J_v$, Cl$^-$ secretion could not substitute for the inhibited HCO$_3^-$ transport. The estimated [HCO$_3^-$] of forskolin or VIP-stimulated fluid was ~ 80 mM.

**Thapsigargin-stimulated $J_v$**—Thapsigargin was used as a surrogate for the natural transmitter acetylcholine because ACh or carbachol cause only transient increases in $I_{sc}$ in Calu-3 cells (39). Thapsigargin increased $J_v$ to 6.4 $\mu l/cm^2/hr$, and this increase was completely eliminated by prior
treatment with bumetanide, consistent with the increased $J_v$ being mediated by $Cl^-$ secretion. However, the pH of fluid stimulated by thapsigargin was 7.23, which suggests that $HCO_3^-$ secretion was not eliminated, but simply became a smaller fraction of anion secretion.

*Combined $J_v$ response to forskolin + thapsigargin*—The largest increases in $J_v$ were produced by combining these two agonists, which gave a mean $J_v$ of 12.2 µl/cm²/hr. This combined response was inhibited 42% by bumetanide and 64% by $HCO_3^-$ replacement with HEPES, and the combination entirely eliminated the response. The pH of fluid secreted in response to these two agonists was 7.4 (23 mM $HCO_3^-$). The interpretation of these results is not direct because of the possibility that inhibition of one anion transport pathway leads to compensatory increases in the other, but given the pH measure, it appears that this condition also represents a combination of $HCO_3^-$ and $Cl^-$ mediated fluid secretion.

*What is the status of CFTR in these conditions?*—Ussing chamber (36) and patch clamp (7) data suggest that CFTR is the only apical anion channel in Calu-3 cells. The presence of basal secretion and the ability of thapsigargin to stimulate secretion are consistent with CFTR being active in the basal state. However, because forskolin alone stimulated secretion and greatly augmented thapsigargin-stimulated secretion, one of two things must be true: (i) CFTR nPo is rate limiting for secretion and not fully active at rest; or (ii) forskolin must stimulate other transport pathways that contribute to $J_v$.

*The pH ($HCO_3^-$ concentration) of secreted fluid*—To summarize the results across all conditions (Fig. 10), the estimated $[HCO_3^-]$ of the secreted fluid was higher than bath for forskolin or VIP stimulation, but was close to bath values during basal secretion and secretion stimulated by thapsigargin alone or in combination with forskolin. These results vary from expectations that forskolin or VIP-mediated secretion will be mediated by nearly pure $HCO_3^-$ secretion and thapsigargin-stimulated secretion by nearly pure $Cl^-$ secretion (4). We hypothesize that the lower than predicted levels of $HCO_3^-$ detected in basal and forskolin-stimulated conditions occurs because of parallel secretion of acid that neutralizes some of the $HCO_3^-$ (29). We further hypothesize that the higher than predicted levels of $HCO_3^-$ observed during thapsigargin-mediated secretion occurs because only a portion of $[HCO_3^-]_i$ is derived from the voltage-
sensitive Na\(^+-\)HCO\(_3\)\(^-\) cotransporter (thapsigargin-induced hyperpolarization makes the transport of anions into the cell less favorable), with the remainder being generated from CO\(_2\) via acetazolamide-sensitive processes that are not voltage-sensitive.

**Comparison with glands**—The thickness of Calu-3 cell monolayers varies from 17-34 \(\mu\)m with an average close to 20 \(\mu\)m (39), so the volume of Calu-3 cells on a 1.1 cm\(^2\) filter is \(\sim 2 \mu\)l. The mean secretion rates observed in this study ranged from 2.7 \(\mu\)l/cm\(^2\)/hr for basal secretion to \(\sim 11 \mu\)l/cm\(^2\)/hr for secretion in response to forskolin and thapsigargin. These rates are equivalent to 120\% and 550\% the estimated Calu-3 cell volume each hr. Direct correlations of human gland submucosal volumes and secretion rates for a selected sample of glands gave average values of 145 nl for gland volume and of 150 nl/hr secretion rate (sustained response to carbachol stimulation), a secretion rate equivalent to \(\sim 100\%\) the gland volume each hour (N. S. Joo, unpublished observations). Although there are many uncertainties in these measures, (the gland volumes include lumens and non-secreting ducts) the comparison suggests that Calu-3 cells are capable of exceeding native gland secretion rates.

**The apical environment in glands and in VG**—Human submucosal glands are multiply branched structures with serous cells occurring at the distal ends of the tubules. The VG is an attempt to reproduce an apical fluid environment that is more similar to that found in the lumens of serous cell tubules in native glands. How close are we?

Direct observations prior to stimulation, as well as images of sections of fixed tissues, indicate that gland tubule lumens may either be closed (empty) or distended to varying degrees. In a typical tubule, the outer diameter is \(\sim 60 \mu\)m, lumen diameter is \(\sim 20 \mu\)m, and cell height is \(\sim 20 \mu\)m (Fig. 12a,b). One way to pose the geometric issues in glands and VG is to consider apical fluid volume relative to cell volume or surface area. Using the observed gland tubule dimensions to calculate volumes (considering tubules as cylinders) shows that the epithelial cell volume for a given length of tubule is \(\sim 8\) times the resting lumen volume (this ratio will increase at the ends of the tubules and of course approaches infinity as the lumen volume approaches zero). In our present experiments, the mean estimated apical volume in the VG experiments was 10 \(\mu\)l, while the cell volume is 2 \(\mu\)l, or 0.2 times the apical fluid volume (Fig. 12c). Thus, in glands the ratio of cell volume to lumen volume is at least a 40-fold greater than in the VG.
With regard to cell surface to apical (or luminal) volume, the cell surface area in the VG is \( \sim 1 \text{ cm}^2 \) \((10^8 \mu\text{m}^2)\) and the apical volume is 10 \( \mu\text{l} \) \((10^4 \text{ nl})\), giving a surface to volume ratio of 10,000 \( \mu\text{m}^2/\text{nl} \). In a gland with a lumen of 20 \( \mu\text{m} \) diameter and 100 \( \mu\text{m} \) length, the apical surface area is \( 6283 \mu\text{m}^2 \) and the lumen volume is \( 31,416 \mu\text{m}^3 \) \((0.031 \text{ nl})\) giving a surface to volume ratio of \( \sim 200,000 \mu\text{m}^2/\text{nl} \). Thus, in gland tubules the ratio of cell surface to lumen volume is about 20 times greater than in the VG.

The conclusion from these considerations is that our apparatus, while achieving a 300-fold improvement over a 3 ml Ussing chamber, nevertheless causes a considerable dilution of the serous cell secretions in comparison with real glands.

**Conclusions**—The virtual gland apparatus provides a new method for direct measurement of fluid secretion by secretory epithelial cell sheets, under conditions that approximate natural conditions found in gland lumens. Initial experiments with the Calu-3 cell line confirm expectations that most fluid secretion is mediated by the electrogenic secretion of \( \text{HCO}_3^- \) and \( \text{Cl}^- \), with forskolin/VIP stimulating a \( \text{HCO}_3^- \)-rich fluid, and thapsigargin, either alone or in combination with forskolin, stimulating a \( \text{Cl}^- \) rich fluid (4). However, secretions never reached the extreme pH values expected from either purely \( \text{HCO}_3^- \) or purely \( \text{Cl}^- \) mediated secretion, and additional work will be required to account for our observations. If Calu-3 cells accurately reflect the properties of gland serous cells, then the acidic pH of gland secretions under all conditions (21, 25) must arise from secondary modifications of the secreted fluid downstream from the serous acini.
ACKNOWLEDGEMENTS

We thank Kimberly Winges and Dennis Lee for help with cell culture and Ramsey Asmar, Teresa Au, Anthony Ko, and Esteban Gomez for contributions to developmental work on the VG apparatus. Present address for Dr. Toshiya Irokawa: Department of Respiratory & Infectious Disease, Post graduate Division, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, JAPAN. Email: <irokawa@int1.med.tohoku.ac.jp>

Grants
This work was supported by National Institutes of Health Grants DK-51817 and HL-60288 and by the Cystic Fibrosis Foundation.

ABBREVIATIONS

The abbreviations used are: 1-EBIO, 1-Ethyl-2-benzimidazolinone; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; DMSO, dimethylsulfoxide; Jv, rate of fluid secretion; KRB, Krebs Ringer Bicarbonate; TEP, transepithelial potential difference; Tg, thapsigargin; VG, virtual gland; VIP, vasoactive intestinal peptide; Fsk, forskolin
Table 1. **Comparison of responses to agonists in VG, Ussing chamber and native glands.**

VG $J_v$ ($\mu$l/cm$^2$/hr), Ussing chamber $I_{sc}$ ($\mu$A/cm$^2$) and gland $J_v$ (nanoliter/min/gland). *Indicates experiments done with cells showing low (< 35 $\mu$A/cm$^2$) basal $I_{sc}$. **The response to thapsigargin (Tg) was from starting values of 0.1 ± 0.05. Thus there was a response, but in absolute terms the response to Tg in native glands is quite small. (n) indicates number of experiments for VG and Ussing and number of animals for gland work. Up to 700 glands were studied. Ussing data is from Krouse, unpublished. Pig gland data is from ref (25).

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<th>VG $\mu$l/cm$^2$/hr</th>
<th>Ussing $\mu$A/cm$^2$</th>
<th>Pig Gland nl/min/gland</th>
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<tr>
<td>Basal (n)</td>
<td>2.4 ± 0.1 (42)</td>
<td>21.2 ± 2 (28)*</td>
<td>0.7 ± 0.1 (56)</td>
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<td>Forskolin (n)</td>
<td>3.5 ± 0.3 (10)</td>
<td>43.6 ± 3.8 (17)*</td>
<td>1.69 ± 0.22 (26)</td>
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<tr>
<td>Tg (n)</td>
<td>5.6 ± 0.8 (5)</td>
<td>ND</td>
<td>0.7 ± 0.2 (4)**</td>
</tr>
<tr>
<td>1-EBIO (n)</td>
<td>7.6 ± 0.9 (5)</td>
<td>172 ± 45 (4)</td>
<td>ND</td>
</tr>
<tr>
<td>Carbachol</td>
<td>ND</td>
<td>ND</td>
<td>2.4 ± 0.2 (12)</td>
</tr>
<tr>
<td>Fsk + Tg (n)</td>
<td>10.3 ± 1 (6)</td>
<td>159 ± 24 (8)</td>
<td>1.9 ± 0.5 (2)</td>
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REFERENCES


Figures

Fig. 1. Illustrations of the virtual gland (VG). The VG comprises three parts, A, the *collection chamber*, B, an unmodified Costar® Snapwell® insert and C, the *bath holder* that is snug-fit into an aluminum jacket of a Peltier-effect temperature control device (details not shown). The holder accepts a thermistor, gas input and electrodes. The collection chamber is made from Costar® Snapwell® with an added plastic barrier that consists of one hole serves as *virtual duct* and another as a *voltage port*. D, VG details showing how the assembly of the Costar filter and the collection chamber creates an *apical chamber* of ~ 10 µl above the cell layer. Secreted fluid flows through the virtual duct into the collection chamber, forming a spherical bubble (*E*, top view, *F*, side view). The bubble expansion is monitored by a digital video camera mounted on a microscope; a 0.5 mm calibration grid is always in the image field. Droplets are periodically collected with constant bore capillaries and preserved between oil blocks for subsequent analysis. A microelectrode in the voltage port (*E*) tracks the transepithelial potential difference. G, Two examples of transepithelial potential difference (TEP) recorded in the VG from different experiments, aligned at the time of drug addition.
Fig. 2. Time course of fluid secretion by Calu-3 cells in the virtual gland. Each point shows the volume of the secreted fluid bubble (in microliters) at the time point indicated. Sudden drops in volume (gray arrows) occur when the fluid was collected. The slope of volume accumulation was converted into instantaneous fluid secretion rates ($J_V$) and that measure is used in subsequent figures.
Fig. 3. Summary of prolonged basal secretion in virtual gland. Time course of basal $J_v$ (filled circles, left axis) and TEP (diamonds, right axis) for five hours. Each point represents means ± SEM for 30 min blocks from 4 experiments.
Fig. 4. Summary of responses to agonists. Each dark bar shows the mean Jv for the indicated condition (left axis), and the adjacent light bar shows the mean TEP (right axis). Error bars indicate SEM. N's for each experiment were: basal: 33, Forskolin: 10, Thapsigargin 5, 1-EBIO 5, Fsk + Tg: 6.
Fig. 5. Time course of responses to agonists and inhibitor. Top graphs show $J_v$ and bottom graphs show the corresponding TEP. $A$, $C$, thapsigargin increased $J_v$ and TEP, and bumetanide inhibited those increases. $B$, $D$, the combination of forskolin and thapsigargin produced the largest increases in $J_v$ and TEP observed in this study (note increased scale of ordinate). Each point represents means ± SEM from 5-6 experiments. Time zero is the point of thapsigargin addition.
Fig. 6. Summary of inhibitory effects on basal $J_v$ and TEP. For each condition the dark bar shows $J_v$ (left axis) and the light bar shows TEP (right axis) measured 30 minutes after drug application, the number of experiments for each condition is shown above each pair of bars. For ouabain both $J_v$ and TEP decline to zero after 90 minutes.
Fig. 7. Responses to forskolin and thapsigargin in the presence of HCO$_3^-$-free solutions. When all HCO$_3^-$ was replaced with HEPES and solutions were gassed with O$_2$, forskolin produced only a brief transient in the TEP and no detectable increase in $J_v$. Subsequent addition of thapsigargin stimulated an increase in both TEP and $J_v$ that was almost as large as normal, but this response was transient.
Fig. 8 Elimination of $J_v$ but not TEP by bumetanide in HCO$_3^-$-free conditions.
Fig. 9. Relationship between \(J_v\) and TEP under various conditions. Each point is the \(J_v\) and TEP (mean \(\pm\) SEM) for data collected as follows. For the basal condition, data were recorded for consecutive 30 min periods from 4 filters, over a period of up to 6 hours of basal secretion for each filter. For the ouabain treatment, data were recorded in ten min blocks after ouabain from 6 filters. For the agonists data were taken from 5-10 filters obtained during ten min sample periods following stimulation. Because \(J_v\) always lagged TEP by \(~10\) min, \(J_v\) was taken at the 10 min period prior to the period used to measure TEP.
Fig. 10. $\text{HCO}_3^-$ concentrations calculated from pH of fluid secreted at various conditions. Each bar shows the mean ± SEM with the number of experiments indicated on the top. The $\text{HCO}_3^-$ concentration was determined by the Henderson-Hasselbalch equation. Dashed line indicates the bath $[\text{HCO}_3^-]$. The value for Fsk + Tg excludes a single outlying experiment in which the calculated $[\text{HCO}_3^-]$ for the secreted fluid was 134 mM.
Fig. 11. Variation of basal pH \((\text{HCO}_3^-\text{-concentration})\) as a function of basal \(J_v\). The least-square fitted line includes an extreme data point that is off the chart: \(J_v\) was 19.1 \(\mu\text{l/cm}^2\text{/hr}\) and calculated \([\text{HCO}_3^-]\) was 107.7 mM.
Fig. 12. Geometrical considerations for apical fluid volumes in glands and VG. 

A, serous tubules from living human submucosal gland imaged with Nomarski optics to show adjacent closed (dashed line) and open (double arrow) lumens. The dimensions of the lumen are obvious when viewed with z axis through focusing.  

B, Cross section of human sero-mucous tubule stained with hematoxylin and eosin to show lumen vs. cell dimensions.  

C, comparison of apical fluid and cells dimensions in VG and serous tubule of submucosal gland. The cell surface to volume ratio is much higher in glands (See text).