J. Membrane Biol. 151, 269–278 (1996)

The Journal of
Membrane
Biology

Swelling and Ca^{2+}-activated Anion Conductances in C127 Epithelial Cells Expressing WT and ΔF508-CFTR

Y. Xia, M.E. Krouse, R. H. Fang, J.J. Wine
Cystic Fibrosis Research Laboratory, Building 420, Jordan Hall, Stanford University, Stanford, CA 94305-2130

Received: 15 November 1995/Revised: 16 February 1996

Abstract. CFTR is a chloride channel that is required for fluid secretion and salt absorption in many exocrine epithelia. Mutations in CFTR cause cystic fibrosis. CFTR expression influences some ion channels, but the range of channels influenced, the mechanism of the interaction and the significance for cystic fibrosis are not known. Possible interactions between CFTR and other ion channels were studied in C127 mouse mammary epithelial cell lines stably transfected with CFTR, ΔF508-CFTR, or vector. Cell lines were compared quantitatively using an ^{125}I efflux assay and qualitatively using whole-cell patch-clamp recording. As expected, ^{125}I efflux was significantly increased by forskolin only in the CFTR line, and forskolin-stimulated whole-cell currents were time- and voltage independent. All three lines responded to hypotonic challenge with large ^{125}I efflux responses of equivalent magnitude, and whole-cell currents were outwardly rectified and inactivated at positive voltages. Unexpectedly, basal ^{125}I efflux was significantly smaller in the ΔF508-CFTR cell line than in either the CFTR or control cell lines ($P < 0.0001$), and the magnitude of the efflux response to ionomycin was largest in the vector cell line and smallest in the cell line expressing ΔF508-CFTR ($P < 0.01$). Whole-cell responses to ionomycin had a linear instantaneous *I-V* relation and activated at depolarizing voltages. Forskolin responses showed simple summation with responses to ionomycin or hypotonic challenge. Thus, we found no evidence for interactions between CFTR and the channels responsible for swelling-mediated responses. Differences were found in basal and ionomycin-stimulated efflux, but these may arise from variations in the clonally selected cell lines that are unrelated to CFTR expression.

Correspondence to: J.J. Wine

Key words: Cystic fibrosis — Forskolin — Efflux — DIDS — DNDS — Chloride channel interactions

Introduction

Mutations in the gene for CFTR (cystic fibrosis transmembrane conductance regulator), a small conductance chloride channel, cause the human genetic disease cystic fibrosis (CF). The loss of CFTR-mediated Cl^- conductance appears to be sufficient to account for most symptoms of CF. However, considerable evidence exists that expression of CFTR influences other cellular properties, including other ion channels.

Two ion channels have been reported to be influenced by CFTR. The amiloride-sensitive epithelial sodium channel (ENaC) has a higher open probability in tissues from CF subjects when compared with normal tissues (Chinet et al., 1994), and this has subsequently been traced to the release from negative regulation of amiloride-blockable epithelial Na^+ channel activity by CFTR (Stutts et al., 1995). To demonstrate this interaction cleanly, both CFTR and ENaC were expressed in MDCK cells (Stutts et al., 1995). An outwardly rectifying chloride channel has also been linked to CFTR expression, but in this case the link is positive (Egan, Schwiebert & Guggino, 1995; Gabriel et al., 1993). To explain the relation, a model has been proposed in which ATP is conducted through open CFTR channels: ATP then binds to apically located purinergic receptors to activate outwardly rectifying Cl^- channels (Schwiebert et al., 1995).

In addition to evidence for a relation between CFTR and these two channels, comparisons of transepithelial transport in CF and normal tissues from both mouse and human establish that Ca^{2+}-activated I_{eq} is increased in tissues where CFTR is defective or missing, and the

Ca^{2+}-activated I_{eq} is reduced by subsequent introduction of CFTR (Johnson et al., 1995). However, the Cl^- channel involved in this kind of transport is not identified, and the interaction could take place at sites in the transport pathway other than the channel. Less work has been done on Cl^- currents activated by cell swelling, but in one study, a two-component current was activated in normal and CF nasal cells by hypotonic solutions, and one of the components was smaller in the CF cells (Chan et al., 1992; Verdon et al., 1995).

As part of an effort to assess pleiotropic effects of CFTR expression, we monitored basal, forskolin-activated, Ca^{2+}-activated and hypotonically-induced ^{125}I efflux in C127 mouse mammary epithelial cells stably transfected with either CFTR or ΔF508-CFTR, *vs.* vector transfected controls. We also obtained whole-cell recordings to determine the electrophysiological signatures of each of the responses. A pilot experiment indicated that the cell lines did not differ for any of these measures, but more extensive measurements detected small but statistically significant differences. However, because of the pattern of results obtained, it is unlikely that these result from the expression of either CFTR or ΔF508-CFTR.

Materials and Methods

Cell Culture

C127 mouse mammary epithelial cells transfected with bovine papilloma virus alone or with a construct including either wild-type or ΔF508 CFTR were developed at Genzyme and are coded BPV2, 2WT2 and 2-508-8, respectively. The cell lines were provided by Jane Amara and Seng Cheng of Genzyme. These cell lines, or similarly transfected lines, have been previously characterized with regard to expression and function of normal and mutant CFTR (Cheng et al., 1995; Marshall et al., 1994; Haws et al., 1996). Cells were grown at 37°C in DME/H21 with 10% FBS (UCSF Cell Culture Facility) in an atmosphere of 5% CO_2 and 95% air. For experiments, cells were grown on 35 mm tissue culture dishes coated with human placental collagen (HPC, Sigma). For efflux experiments, the cells were grown to confluency. For patch-clamp experiments the cells were plated less densely and were usually patched as isolated cells.

Isotopic Flux Studies

The methods used are based on Venglarik, Bridges & Frizzell (1990), and our modifications have been described in detail (Haws et al., 1994; Krouse et al., 1994; Luckie et al., 1994). In brief, efflux buffer was (in mM): 10 N-2-hydroxy ethylpiperazine-N′-2-ethane sulfonic acid (HEPES), 5.4 KCl, 130 NaCl, 1.8 $CaCl_2$, 1.0 Sodium phosphate (monobasic), 0.8 $MgSO_4$, pH adjusted to 7.4 with NaOH, and glucose 100 mg/100 ml. Cells were incubated at 37°C for 2 hr in efflux buffer containing ~2 μCi of ^{125}I/ml, then washed 3× with 1 ml aliquots of 22°C buffer. Efflux samples were collected at 30-sec intervals with total fluid replacement. Remaining counts were removed by lysing cells, scintillation fluid was added, and samples were counted in a Beckman liquid scintillation counter. Efflux rate constants were estimated according to the formula given by Venglarik et al. (1990). A problem with this method is that the estimated efflux rate is influenced by the sampling interval, such that longer intervals result in an apparently lower efflux rate. This may occur because of increasing influx of tracer from unstirred layers at longer time intervals. To avoid this problem, we used a constant 30-sec interval in all experiments except for one extended experiment that required 1-min sampling intervals (Fig. 5*C*,*F*).

Patch-clamp Recording

Whole-cell currents were recorded with an Axopatch 1C amplifier (Axon Instruments), digitized (PCM-2, Medical Systems) and stored on videotape and sometimes on hard disk using the program pClamp (Axon Instruments). All patch-clamp recordings were made at 20–23°C. Electrodes were pulled from very soft glass capillaries (LA16, Dagan Corporation) and coated with Sylgard©. After heat polishing, resistances were 2–5 MΩ in the solutions listed below. The standard bath solution was (in mM): NaCl 150, $CaCl_2$ 2.5, $MgCl_2$ 2.5, and HEPES 10. The pH was adjusted to 7.3 with NaOH and osmolarity was 300 mOsm/L. The standard pipette solution was (in mM): CsCl 125, HEPES 10, $MgCl_2$ 2 and EGTA 2. The calculated free Ca^{2+} concentration was < 10 nM, pH was adjusted to 7.3 with CsOH and osmolarity 240 mOsm/L. The osmolarity difference of 60 mOsm/L between pipette and bath solutions minimizes swelling-induced currents (Worrell et al., 1989). For all cells, 3 mM Mg-ATP (prepared fresh daily) was added to the pipette solution. Initial cell-attached seal resistances were > 10 GΩ, usually adequate to attempt going whole cell. Whole-cell capacitance was electronically compensated prior to data recording where possible and no leak subtraction was used. Series resistance was ~10 MΩ for all cell types; compensation was typically set at 80%, resulting in, at most, a 5% error in the clamp voltage for cells with largest currents. The first trial began after series and capacitance compensation and marks the zero time of the experiment.

Reagents

Mg^{2+}-ATP was obtained from Sigma; 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) from Pfaltz & Bauer or Calbiochem, Ultima gold scintillation fluid from Packard, ^{125}I from ICN. ATP, DNDS and DIDS were dissolved in water as required.

Statistics

Except where noted, data are reported as means ± SD. Statistical significance was assessed with the statistical packages of Microsoft Excel.

Results

Basal ^{125}I Efflux Rates Were Significantly Lower in the 2-508-8 Line

Basal efflux rates were compared for a 2–4-min efflux period prior to stimulation. All dishes used in these experiments contributed to this measurement, providing the largest *n* for any experiment. As shown in Fig. 1 (open bars), basal efflux did not differ significantly between the 2WT2 and BPV2 cells lines, but basal efflux from the

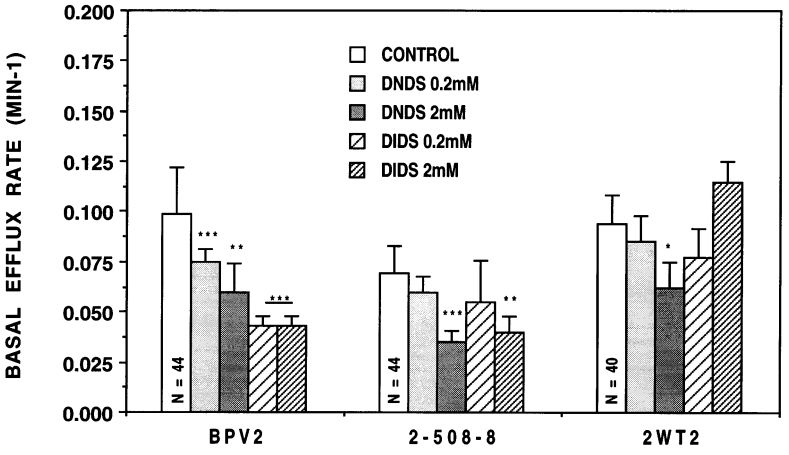

Fig. 1. Basal efflux rates of ^{125}I for three cell lines. The first (open) bar in each set of histograms shows the mean ± SD efflux rate (min^{-1}) at the 3-min time point for 40–44 replicates of basal efflux during the first 2–4 min of the efflux period. The rate for 2-508-8 cells was significantly lower than either of the other two lines ($P < 0.0001$). The following 4 bars indicate the effects of 0.2 and 2 mM DNDS or DIDS on basal efflux rates ($n = 4$ for each condition). Significance of difference between control and inhibitor is indicated by asterisks: *$P < 0.01$, **$P < 0.001$ and ***$P < 0.0001$, (two-tailed Student's *t*-test).

2-508-8 line was significantly reduced relative to either of the other two lines ($P < 0.0001$, $n = 40$). These results depart from the simple hypothesis that cells expressing CFTR might display increased efflux because they express an additional anion conductance pathway. To determine the extent to which basal ^{125}I flux was occurring through CFTR channels, cells were exposed to two concentrations of DNDS and DIDS, which inhibit a broad range of anion channels (Bridges et al., 1989; Greger, 1990; Singh et al., 1991; Venglarik, Singh & Bridges, 1994) but do not inhibit CFTR (Cliff, Schoumacher & Frizzell, 1992). As shown in Fig. 1 (patterned bars), efflux from the BPV2 line was significantly inhibited by all concentrations of DNDS and DIDS, but only the higher concentrations of stilbenes were effective in 2-508-8 cells. In 2WT2 cells, only the higher level of DNDS was effective. The higher level of DIDS actually caused a small, but significant increase of efflux.

Whole-cell Patch Clamp Currents had the Expected Signatures in Vector Control and 2WT2 Cells

To establish that the efflux responses are mediated by anion channels, and to determine the signatures of the chloride conductance increases, we measured Cl^- currents with the whole-cell patch clamp method in the control BPV2 cell line and the 2WT2 line. (These currents are too variable, and the procedure too tedious, to obtain significant data on the relative magnitude of the responses.)

Forskolin (10 μM) had no effect on the BPV2 cell line (Fig. 2*A*, left) but produced large, time and voltage-independent conductance in the 2WT2 cells (Fig. 2*A*, right), a signature characteristic of CFTR-mediated currents. Under the conditions of our experiments, no additional currents were observed, indicating that the forskolin-induced response was produced exclusively by currents through CFTR channels.

In both cell lines, ionomycin produced currents with a linear instantaneous *I-V* plot; the currents activated at voltages more positive than ~20 mV and inactivated at more negative voltages (Fig. 2*B*). The time course for activation in cells of either type could be fitted with a single exponential having a time constant of ~250–350 msec.

Hypotonic bath solutions (50% of normal osmolarity) produced dramatic increases in an outwardly rectifying whole-cell chloride current that inactivated at voltages more positive than +50 mV (Fig. 2*C*). The inactivating currents in either cell type could be fit with two exponentials having time constants of ~900 and ~100 msec at +100 mV (Solc & Wine, 1991; Worrell et al., 1989).

Forskolin Stimulated ^{125}I Efflux only from 2WT2 Cells

The response to forskolin stimulation was expected to differ qualitatively among the cell lines, based on past work and on the results from the preceding whole-cell experiments. As expected, the forskolin simulated ^{125}I efflux was large in CFTR-transfected cells and lacking in control cells. Cells transfected with ΔF508 showed a negligible increase to forskolin within the time scale used in these experiments (Fig. 3, forskolin).

Ionomycin-stimulated ^{125}I Efflux was Largest in BPV2 Cells and Smallest in 2-508 Cells

Comparisons of certain epithelia in normal and CFTR ''knockout'' mice (Grubb, Paradiso & Boucher, 1994), indicate that Ca^{2+}-mediated I_{sc} is increased in mice in which the CFTR gene is disrupted. In an attempt to assess the generality of that finding, we compared ionomycin-induced efflux in the three C127 cell lines. Efflux induced by ionomycin (1.3 and 13 μM) was large in all

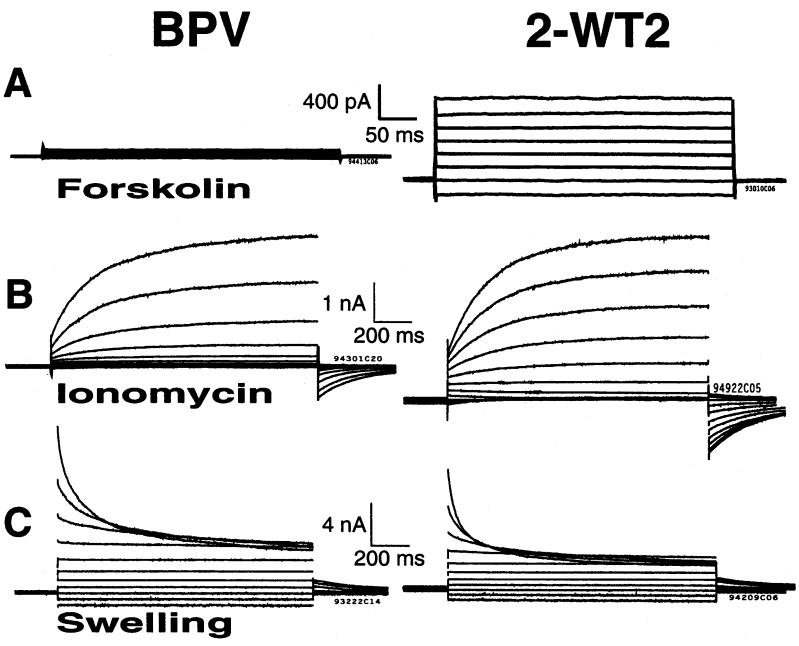

Fig. 2. Representative whole-cell currents in response to forskolin, ionomycin, and hypotonic challenge in vector control (BPV2) and CFTR expressing (2WT2) cells. (*A*) Currents produced by voltage steps from a holding potential of −50 to +100 mV, decreasing by 25 mV steps to −75 mV, taken at the point when maximum currents were observed after preincubation with 10 μM Forskolin. (*B* and *C*) Currents produced by voltage steps from a holding potential of −50 to +110 mV decreasing in 20 mV steps to −90 mV. (*B*) Maximum currents produced by preincubation with 1 μM Ionomycin. (*C*) Maximum currents produced by a bath dilution of 50%. The pipette contained 3 mM ATP for all whole-cell recordings (for other solutions *see* methods).

three cell lines (Fig. 3, ionomycin), but the line expressing ΔF508-CFTR had a response only about half that of the vector control and slightly but significantly smaller than the response of the line expressing CFTR. The time courses of the responses were indistinguishable in the three cell lines. These results do not fit with any simple hypothesis of CFTR interactions with Ca^{2+}-activated Cl^- channels, suggesting that differences among the cell lines other than CFTR expression are responsible for the observed patterns.

Hypotonic Shock

Prior reports suggest that CFTR might interact with volume-regulated chloride channels (Chan, Goldstein & Nelson, 1992; Valverde, 1995). To test for that possibility, each cell line was challenged with bath dilutions of 25% and 50%. Efflux responses induced by 50% bath dilution were very large in all three cell lines, while the responses to a 25% dilution were just above threshold in all three lines (Fig. 3, hypotonicity). Thus, neither the threshold nor magnitude of the response to cell swelling was altered by expression of either form of CFTR.

Responses to Ionomycin and Hypotonicity Were Inhibited by Stilbenes Equally in all Three Cell Lines

One possible explanation for response differences to ionomycin among the cell lines is that additional types of anion channel might be recruited or repressed by expression of CFTR or ΔF508-CFTR. To help reveal such a possibility, the cells were stimulated either in normal efflux buffer or in the presence of 200 μM or 2 mM DIDS or DNDS. As shown in Fig. 4*A*, the Ca^{2+}-mediated responses were inhibited to the same extent in all three cell lines. As a control, the equivalent responses to hypotonic medium were also tested and, as expected, were also inhibited to an equivalent extent (Fig. 4*B*).

Interactions of Forskolin with Ionomycin and Swelling-activated Responses

In the experiments to this point, CFTR channel activity was presumably at a low level unless stimulated with forskolin. However, interactions between CFTR and outwardly rectifying chloride channels (Schwiebert et al., 1995) and between CFTR and sodium channels (Chinet et al., 1994) require CFTR channel activity. Therefore, interactions among CFTR and Ca^{2+}- and swelling-activated chloride channels might also require activation of CFTR. To assess that possibility, we compared the responses to swelling and ionomycin following forskolin stimulation in all three cell lines. The results are shown in Fig. 5 and Table 2. In the control BPV2 cell line, responses to ionomycin and hypotonic shock were essentially identical in the presence of forskolin (Fig. 5*A, D*). The same was true for the 2-508-8 cell line (Fig. 5*C, F*). (Note that for this cell line, forskolin was added 8 min prior to ionomycin or hypotonic shock because of evidence that activation of ΔF508-CFTR is considerably delayed relative to wild-type (Dalemans et al., 1991; Drumm et al., 1991; Haws et al., 1996).) For 2WT2 cells, forskolin activated a current via CFTR channels.

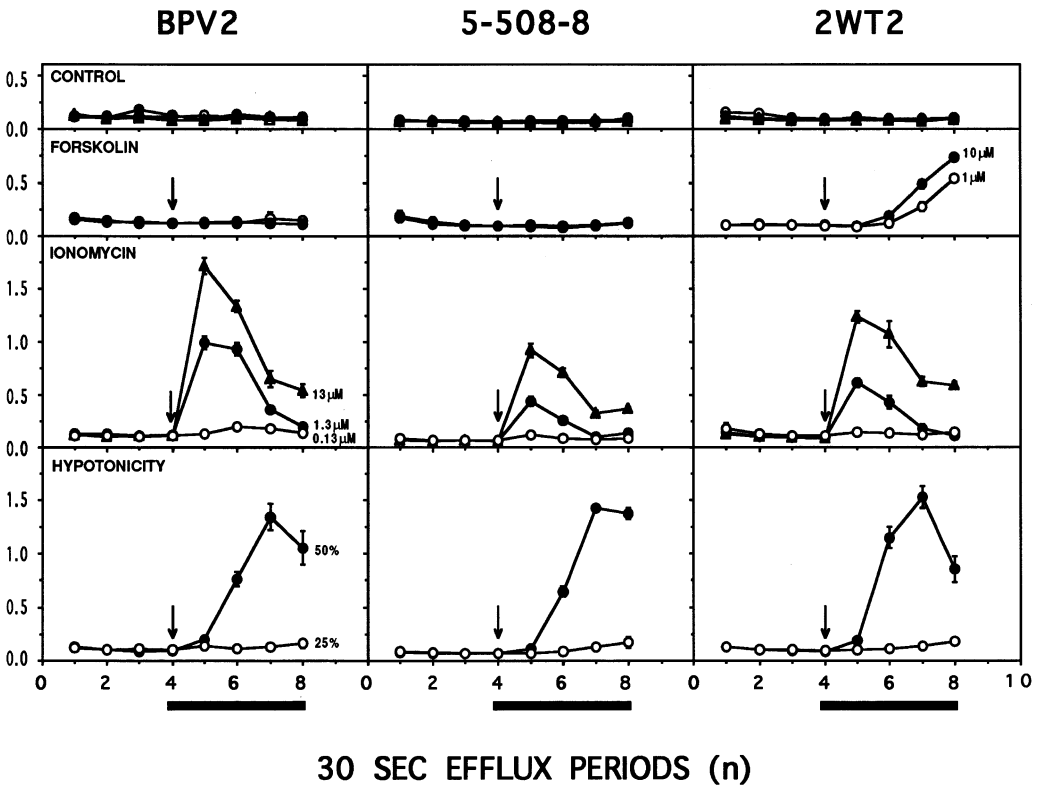

Fig. 3. Dose-response relations for forskolin, hypotonic buffer and ionomycin in vector control, ΔF508 and WT CFTR cell lines. Cells were tested with each agonist or with buffer dilution in the amounts indicated. Each point is the mean ± SD estimated efflux rate constant for a 30-sec interval for the indicated number of 35 mm dishes of cells (*see* Table 1). Agonists or dilutions were indicated by arrows. (*A*) Control (basal efflux). (*B*) Responses to forskolin (1 and 10 μM). 2WT2 responses *vs.* other two cell types differed significantly ($P < 0.01$). (*C*) Responses to ionomycin (0.13, 1.3 and 13 μM). Differences in peak responses to 13 μM ionomycin differed significantly among all three cell lines ($P < 0.005$). (*D*) Responses to 25 and 50% dilutions of the bath solution with distilled water. Responses among cell lines did not differ significantly.

In the absence of specific interactions among the channels this forskolin-induced current should simply be additive with the other two currents, and that was observed (Fig. 5*B, E*). To obtain the data in Table 2, the areas under each response curve was measured and the results compared to computed area-under-the curve values based on summation. As shown, the ratios between measured and computed responses fell within the range of 0.90 to 1.15, suggesting that any interaction beyond simple summation was very small.

Discussion

A summary of our results is shown in Fig. 6. We observed two distinct types of native Cl^- currents in the C127 mouse epithelioid cells: a Ca^{2+}-dependent current that activated at positive voltages, and a swelling-activated current that inactivated at positive voltages. Each of these currents was inhibited by stilbenes, which serves to distinguish them from CFTR currents. The question we set out to answer was whether CFTR expression might influence either of these currents, as assessed with ^{125}I efflux. While this simple question did not result in an unequivocal answer, we favor the conclusion that CFTR expression did not influence either of the channels we assessed, and that the small difference observed in the 2-508-8 line resulted from clonal selection. We discuss the results in order of increasing complexity.

Hypotonic Solutions Produced Equivalent ^{125}I Efflux in all Cell Lines

The swelling-activated current we observed in BPV2 and 2WT2 cells is ubiquitous in cultured cells, and arises from a channel termed VSOAC, for *v*oltage *s*ensitive *o*smolyte and *a*nion *c*hannel (Jackson, Morrison & Strange, 1994). VSOAC is a channel of ~50 pS that is normally open at negative potentials and rapidly inactivates at potentials more positive than ~60 mV (e.g., Solc

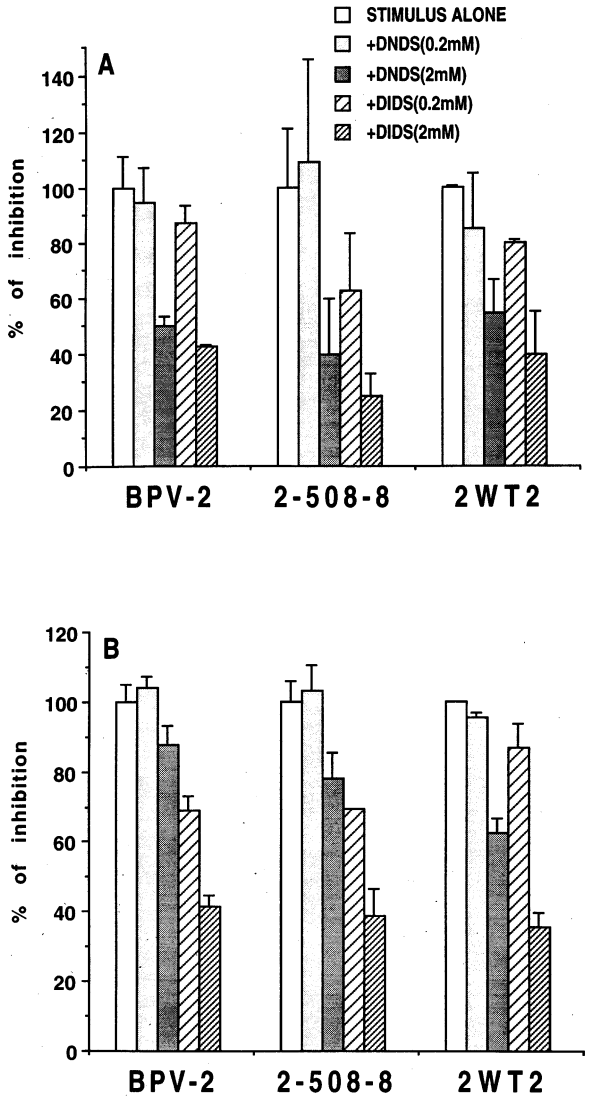

Fig. 4. Responses to ionomycin and hypotonicity were inhibited by stilbenes equally in all three cell lines. (*A*) Normalized responses of the three cell lines to ionomycin following pretreatment with 0.2 or 2 mM DNDS or DIDS. (*B*) Normalized responses of the three cell lines to 50% bath dilution after pretreatment with 0.2 or 2 mM DNDS or DIDS.

& Wine, 1991). Thus, VSOAC channels have voltage-dependent properties essentially opposite to those of the calcium-dependent channels. Chan et al. (1992) compared VSOAC current in human nasal cells from normal and CF individuals and found no difference in the current. In a comparison of cell volume regulation in intestinal crypts of normal and CF mice, Valverde et al. (1995) did find that volume regulation of crypt cells was defective in the CF mice, but they attribute this to a loss of volume-activated K^+ conductance. We did not assess K^+ conductances in these cells, and an altered K^+ conductance would not be expected to have influenced the ^{125}I fluxes (Luckie et al., 1994). Thus, all studies to date indicate no interaction between CFTR and VSOAC.

CFTR Imparted Forskolin-stimulated Efflux and Whole Cell Cl^- Conductance Increase, but This Did Not Interact with the Other Two Cl^- Conductances

We confirmed that CFTR expression confers a marked, forskolin-activated Cl^- conductance that is absent in non-transfected cells (Cheng et al., 1995; Marshall et al., 1994; Dechecchi et al., 1993; Haws et al., 1996).[1] In some models of how CFTR influences other channels, CFTR must first be activated to have an effect (e.g., Schwiebert et al., 1995). Therefore, we looked for interactions between activated CFTR and the chloride currents mediated by Ca^{2+} and VSOAC, but no interactions were observed. The lack of interactions in our experiments could mean that these mouse cells lack elements required for the interactions, or possess elements that suppress the interactions. Also, because we used ionomycin to stimulate Ca^{2+}-activated Cl^- channels, we would miss any effects that CFTR might exert on earlier stages in the stimulus-response pathway (Reinlib et al., 1992). Finally, the Ca^{2+}-dependent Cl^- current we studied has not been shown to be the current responsible for Ca^{2+}-stimulated I_{eq} in intact epithelia (*see below*). Therefore, it may be that these Ca^{2+}-activated channels are not influenced by CFTR in any circumstances.

Differences in Basal Efflux Among the Cell Lines

Basal efflux from the 2-508-8 cell line was significantly lower than either of the other cell lines. However, although the difference was highly significant, it was extremely small in magnitude. DNDS (2 mM) inhibited ~50% of the basal efflux rate in all three lines, suggesting that similar proportions of Ca^{2+}-dependent and/or swelling-activated channels were active at rest in all cell lines, i.e., CFTR appears to contribute little to basal efflux. If the basal efflux rate occurs via Ca^{2+}-dependent chloride channels, the lower rate observed in the 2-508-8 line would be consistent with the lower efflux in that line in response to ionomycin (*see below*). DIDS (2 mM) also inhibited the BPV2 and 2-508-8 cells, but in contrast with DNDS, it actually increased efflux in 2WT2 cells ($P < 0.02$). The differential DIDS effect may indicate that DIDS is elevating cytosolic Ca^{2+} in these cell lines, as it

[1] Under the conditions of the present experiments, chloride conductance was virtually absent in the 2-508-8 cell line as well, but we and others have shown elsewhere that a significant level of response can be observed in this line under appropriate conditions (Haws et al., 1996; Dechecchi et al., 1993; Cheng et al., 1995).

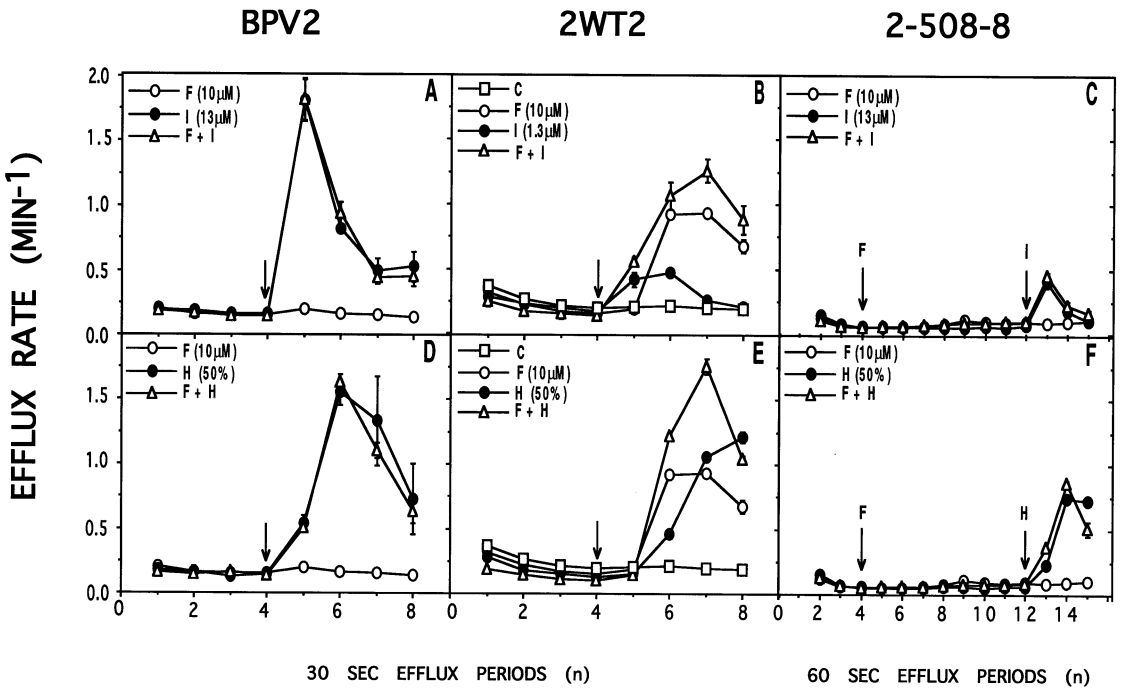

Fig. 5. Efflux responses to combinations of forskolin with ionomycin and hypotonic shock. Each panel shows three traces: the response to forskolin, the response to either ionomycin (*A–C*), or to hypotonic bath solution (*D–F*), and the combined response to forskolin and either ionomycin or hypotonic bath solution. For 2WT2 cells, an additional control trace (no stimulus) is shown. This trace was omitted for the other two lines because it was indistinguishable from the forskolin traces. In each panel the arrow indicates the time of drug addition. Drugs were added together except for 2-508-8 cells, where ionomycin addition was delayed to coincide with the peak response to forskolin, which is considerably delayed compared with WT cells (*see text*). Because of the delayed response, the efflux periods in this experiment were 60 sec instead of 30 sec, resulting in a reduction in the apparent efflux rate constant (*see* Materials and Methods). At this scale, the very small response to forskolin in 2-508-8 cells is not apparent (*see* footnote).

	Basal	Forskolin	Calcium	Swelling
2-508-8	↓(−30%)	=	↓(−46%)	=
2WT2	=	↑(+567%)	↓(−28%)	=

Fig.6. Summary of Results. The relations shown indicate comparisons between the cell lines shown and the control (BPV) cell line. ↑:larger., ↓:smaller., =:equal.

was previously shown to do for T84 cells (Brayden et al., 1993). If DIDS does elevate Ca^{2+} in C127 cells, it could increase efflux via PKC activation of CFTR, as has been demonstrated previously for 2WT2 cells (Dechecci et al., 1993). However, we did not observe a contribution of CFTR currents to whole-cell responses evoked by ionomycin (Fig. 2*B*).

Responses to Ionomycin

In our experiments, Ca^{2+}-dependent efflux responses were significantly smaller in 2WT2 cells, and, unexpectedly, were smallest in the 2-508 cell line (Fig. 3 and Table 1). Again, although these differences were significant, their magnitude was small. The smaller response in 2WT2 cells is reminiscent of the complementarity between CFTR and Ca^{2+}-activated I_{eq} responses observed in comparisons of confluent, primary epithelia of normal and CF human and mouse tissues (Grubb et al., 1994; Clarke et al., 1994; Johnson et al., 1995). However, there are too many differences between the I_{eq} responses observed in polarized cell sheets and the responses observed in these nonpolarized cells to conclude that a similar mechanism is operating in each instance. Such a possibility is made more remote by the unexpected finding that the line expressing ΔF508-CFTR has an even smaller Ca^{2+}-dependent response than either CFTR or vector control cells — an observation at odds with the human data. Also, in other experiments with single, unpolarized cells from the pancreas of normal and CF mice, Gray et al. (1994) found no significant differences in Ca^{2+}-dependent current.

The biggest difference in Ca^{2+}-dependent efflux was between the BPV2 (vector transfected) and 2-508-8 (ΔF508-CFTR transfected) cell lines, with 2WT2 cells (CFTR transfected) being intermediate. This is difficult to explain by most present models of CFTR function, but

Table 1. Relations among the three cell types for various measurments

Measurement	Relative response magnitude					*Significance
Basal efflux rates	2WT2		BPV		508	$P < 0.0001$,
	0.094 ± 0.014	=	0.099 ± 0.023	>*	0.069 ± 0.014	$n = 40$
Stilbene inhibition, basal efflux	BPV	>	508	>	2WT2	*see* Fig. 1
Forskolin stimulation, efflux	2WT2		508		BPV	$P < 0.01$,
(10 μM, peak rate)	0.734 ± 0.020	>*	0.126 ± 0.022	=	0.110 ± 0.006	$n = 4$
Ca^{2+} stimulation, efflux	BPV		2WT2		508	$P < 0.005$,
(13 μM, peak rate)	1.719 ± 0.075	>*	1.237 ± 0.057	>*	0.921 ± 0.062	$n = 4$
Hypotonic stimulated efflux	BPV		508		2WT2	NS
(50%, peak rate)	1.346 ± 0.12	=	1.431 ± 0.017	=	1.530 ± 0.100	$n = 4$
Stilbene inhibition of						NS
stimulated efflux	2WT2	=	508	=	BPV	*see* Fig. 4

All differences are shown, but only those indicated by ''>*'' signs were significant.

Table 2. Absence of interactions between efflux responses to forskolin, ionomycin, and hypotonic challenge

	BPV2	2-508-8	2WT2
Forskolin (10 μM)	0.02 ± 0.01	0.27 ± 0.002	0.915 ± 0.027
IM (1.3 or 13 μM)	1.42 ± 0.10	0.515 ± 0.017	0.326 ± 0.034*
Hypotonic (50%)	1.636 ± 0.140	1.238 ± 0.021	0.913 ± 0.012
Predicted (F + IM)	1.44	0.785	1.241*
Observed (F + IM)	1.45 ± 0.141	0.838 ± 0.040	1.423 ± 0.087*
Observed/predicted (F + IM)	1.01	1.07	1.15
Predicted (F + SW)	1.66	1.508	1.828
Observed (F + SW)	1.54 ± 0.04	1.497 ± 0.005	1.643 ± 0.022
Observed/predicted (F + SW)	0.93	0.99	0.90

Numerical data from Fig. 5 are expressed as average Δ for Area Under Curve (AUC), of each trace (x = min, y = efflux rate) after drug addition, ÷ AUC for basal efflux trace of the corresponding time segment, ± SD. Predicted responses were calculated assuming perfect summation, and were compared with actual values obtained. The ratio of actual to predicted responses did not differ significantly from 1 for any combination of stimuli and cell types.
*IM = 1.3 μM, the rest are all 13 μM.

some authors propose that the internal *presence* of ΔF508-CFTR causes specific defects. For example, using the same cell lines studied here, Dosanjh et al. (1994) found that BPV2 and 2WT2 cells had almost identical patterns of cell-surface staining with elderberry bark lectin, while 2-508-8 cells had virtually no staining. They attributed this difference to the expression of ΔF508-CFTR in that cell line. By the same logic, we could propose that something about ΔF508-CFTR expression is interfering with Ca^{2+}-dependent ^{125}I efflux.

However, such a mechanism suggests a dominant mode of expression for the phenotype. That is in contrast with cystic fibrosis symptoms, which are recessive in most organs and intermediate or ''codominant'' in some organs, such as the adrenergically stimulated sweat gland (Behm et al., 1987) and possibly the crypt cells of the intestine (Gabriel et al., 1993), *see* Wine (1988) for a general discussion of CF heterozygosity. Furthermore, the best present evidence is that the fundamental difference between cells expressing WT and ΔF508-CFTR is that a much larger proportion (~99%) of ΔF508-CFTR is rapidly degraded by a ubiquitin-proteasome pathway, while for WT CFTR only ~75% is degraded by this same pathway with the same kinetics (Ward & Kopito, 1994, and Ward, Omura, & Kopito, 1995; Jensen et al., 1995). It thus seems more reasonable to attribute differences among WT and ΔF508-CFTR cells to the much larger proportion of functional CFTR in the plasma membrane of WT cells. Therefore, we favor the hypothesis that these clonally selected cell lines differ in features other than those for which they were specifically selected. If experiments like these find no differences among cell lines (as was the case with the swelling-activated currents), the interaction hypothesis is not supported, but if differences are found, they are uninterpretable unless a group of different, clonally selected cell lines are tested.

In conclusion, the differences we observed in Ca^{2+}-dependent and basal efflux among the three cell lines are probably not related to CFTR function, since no interactions were observed when CFTR was activated, and the most divergent line with regard to Ca^{2+}-dependent responses had the mutant form of CFTR, the vast majority of which does not reach the plasma membrane (Cheng et al., 1990, 1995). CFTR expression can clearly influence other ion channels, but proof of such influence requires evidence beyond a comparison of two clonally selected cell lines.

We thank Seng Cheng and Jane Amara of Genzyme for providing the C127 cell lines and information about growing them, and Tina Law and Ilynn Nepomuceno for cell culture. Supported by HL42368 and DK45913, CFRI, Cystic Fibrosis Foundation, Patricia Bresee and Ron and Kay Presnell.

References

Behm, J.K., Hagiwara, G., Lewiston, N.J., Quinton, P.M., Wine, J.J. 1987. Hyposecretion of beta-adrenergically induced sweating in cystic fibrosis heterozygotes. *Ped. Res.* **22:**271–276

Brayden, D.J., Krouse, M.E., Law, T., Wine, J.J. 1993. Stilbenes stimulate T84 Cl-secretion by elevating Ca^{2+}. *Am. J. Physiol.* **264:**G325–G333

Bridges, R.J., Worrell, R.T., Frizzell, R.A., Benos, D.J. 1989. Stilbene disulfonate blockade of colonic secretory Cl^- Channels in planar lipia bilayers. *Am. J. Physiol.* **256:**C902-C912

Chan, H.C., Goldstein, J., Nelson, D.J. 1992. Alternate pathways for chloride conductance activation in normal and cystic fibrosis airway epithelial cells. *Am. J. Physiol.* **262:**C1273–C1283

Cheng, S.H., Fang, S.L., Zabner, J., Marshall, J., Piraino, S., Schiavi, S.C., Jefferson, D.M., Welsh, M.J., Smith, A.E. 1995. Functional activation of the cystic fibrosis trafficking mutant delta F508-CFTR by overexpression. *Am. J. Physiol.* **268:**L615–L624

Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan, C.R., Smith, A.E. 1990. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* **63:**827–834

Chinet, T.C., Fullton, J.M., Yankaskas, J.R., Boucher, R.C., Stutts, M.J. 1994. Mechanism of sodium hyperabsorption in cultured cystic fibrosis nasal epithelium: a patch-clamp study. *Am. J. Physiol.* **266:**C1061–C1068

Clark, L.L., Grubb, B.R., Yankaskas, J.R., Cotton, C.U., McKenzie, A., Boucher, R.C. 1994. Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in Cftr(-/-) mice. *Proc. Natl. Acad. Sci. USA* **91:**479–483

Cliff, W.H., Schoumacher, R.A., Frizzell, R.A. 1992. cAMP-activated Cl channels in CFTR-transfected cystic fibrosis pancreatic epithelial cells. *Am. J. Physiol.* **262:**C1154–C1160

Dalemans, W., Barbry, P., Champigny, G., Jallat, S., Dott, K., Dreyer, D., Crystal, R.G., Pavirani, A., Lecocq, J.P., Lazdunski, M. 1991. Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. *Nature* **354:**526–528

Dechecchi, M.C., Tamanini, A., Berton, G., Cabrini, G. 1993. Protein Kinase C activated chloride conductance in C127 cells stably expressing the cystic fibrosis gene. *J. Bio. Chem.* **268:**11321–11325

Dosanjh, A., Lencer, W., Brown, D., Ausiello, D.A., Stow, J.L. 1994. Heterologous expression of delta F508 CFTR results in decreased sialylation of membrane glycoconjugates. *Am. J. Physiol.* **266:**C360–C366

Drumm, M.L., Wilkinson, D.J., Smit, L.S., Worrell, R.T., Strong, T.V., Frizzell, R.A., Dawson, D.C., Collins, F.S. 1991. Chloride conductance expressed by delta F508 and other mutant CFTRs in *Xenopus* oocytes. *Science* **254:**1797–1799

Egan, M.E., Schwiebert, E.M., Guggino, W.B. 1995. Differential expression of ORCC and CFTR induced by low temperature in CF airway epithelial cells. *Am. J. Physiol.* **268:**C243–C251

Gabriel, S.E., Clarke, L.L., Boucher, R.C., Stutts, M.J. 1993. CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship. *Nature* **363:**263–268

Gray, M.A., Winpenny, J.P., Porteous, D.J., Dorin, J.R., Argent, B.E. 1994. CFTR and calcium-activated chloride currents in pancreatic duct cells of a transgenic CF mouse. *Am. J. Physiol.* **266:**C213–C221

Greger, R. 1990. Chloride channel blockers. *Meth. Enz.* **191:**793–810

Grubb, B.R., Paradiso, A.M., Boucher, R.C. 1994. Anomalies in ion transport in CF mouse tracheal epithelium. *Am. J. Physiol.* **267:**C293–C300

Haws, C., Finkbeiner, W.E., Widdicombe, J.H., Wine, J.J. 1994. CFTR in Calu-3 human airway cells: channel properties and role in cAMP-activated Cl− conductance. *Am. J. Physiol.* **266:**L502–L512

Haws, C., Nepomuceno, I., Krouse, M.E., Wakelee, H., Law, T., Xia, Y., Nguyen, H., Wine, J.J. ΔF508-CFTR channels: kinetics, activation by forskolin and potentiation by xanthines. *Am. J. Physiol.* (*in press*)

Jackson, P.S., Morrison, R., Strange, K. 1994. The volume-sensitive organic osmolyte-anion channel VSOAC is regulated nonhydrolytic ATP binding. *Am. J. Physiol.* **267:**C1203–C1209

Jensen, T.J., Loo, M.A., Pind, S., Williams, D.B., Goldberg, A.L., Riordan, J.R. 1995. Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* **83:**129–135

Johnson, L.G., Boyles, S.E., Wilson, J., Boucher, R.C. 1995. Normalization of raised sodium absorption and raised calcium-mediated chloride secretion by adenovirus-mediated expression of cystic fibrosis transmembrane conductance regulator in primary human cystic fibrosis airway epithelial cells. *J. Clin. Invest.* **95:**1377–1382

Krouse, M.E., Haws, C.M., Xia, Y., Fang, R.H., Wine, J.J. 1994. Dissociation of depolarization-activated and swelling-activated Cl− channels. *Am. J. Physiol.* **267:**C642–C649

Luckie, D.B., Krouse, M.E., Harper, K.L., Law, T.C., Wine, J.J. 1994. Selection for MDR1/P-glycoprotein enhances swelling-activated K^+ and Cl^- currents in NIH/3T3 cells. *Am. J. Physiol.* **267:**C650–C658

Marshall, J., Fang, S., Ostedgard, L.S., O'Riordan, C.R., Ferrara, D., Amara, J.F., Hoppe, H. 4th., Scheule, R.K., Welsh, M.J., Smith, A.E. 1994. Stoichiometry of recombinant cystic fibrosis transmembrane conductance regulator in epithelial cells and its functional reconstitution into cells in vitro. *J. Bio. Chem.* **269:**2987–2995

Reinlib, L., Jefferson, D.J., Marini, F.C., Donowitz, M. 1992. Abnormal secretagogue-induced intracellular free Ca^{2+} regulation in cystic fibrosis nasal epithelial cells. *Proc. Nat. Acad. Sci. USA* **89:**2955–2959

Schwiebert, E.M., Egan, M.E., Hwang, T.H., Fulmer, S.B., Allen, S.S., Cutting, G.R., Guggino, W.B. 1995. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* **81:**1063–1073

Singh, A.K., Afink, G.B., Venglarik, C.J., Wang, R.P., Bridges, R.J. 1991. Colonic Cl channel blockade by three classes of compounds. *Am. J. Physiol.* **261:**C51–C63

Solc, C.K., Wine, J.J. 1991. Swelling-induced and depolarization-induced Cl-channels in normal and cystic fibrosis epithelial cells. *Am. J. Physiol.* **261:**C658–C674

Stutts, M.J., Canessa, C.M., Olsen, J.C., Hamrick, M., Cohn, J.A., Rossier, B.C., Boucher, R.C. 1995. CFTR as a cAMP-dependent regulator of sodium channels. *Science* **269:**847–850

Valverde, M.A., O. Brien, J.A., Sepulveda, F.V., Ratcliff, R.A., Evans, M.J., Colledge, W.H. 1995. Impaired cell volume regulation in intestinal crypt epithelia of cystic fibrosis mice. *Proc. Natl. Acad. Sci.* **92:**9038–9041

Venglarik, C.J., Bridges, R.J., Frizzell, R.A. 1990. A simple assay for agonist-regulated Cl and K conductances in salt-secreting epithelial cells. *Am. J. Physiol.* **259:**C358–C364

Venglarik, C.J., Singh, A.K., Bridges, R.J. 1994. Comparison of -nitro versus -amino 4,4′-substituents of disulfonic stilbenes as chloride channel blockers. *Mol. Cell. Biochem.* **140:**137–146

Verdon, B., Gray, M.A., Evans, M.J., Colledge, W.H., Ratcliff, R., Argent, B.E. 1995. CFTR does not regulate volume-sensitive Cl channels in mouse pancreatic duct cells. *Ped. Pulmonol.* **Suppl. 12:**196

Ward, C.L., Kopito, R.R. 1994. Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *J. Bio. Chem.* **269:**25710–25718

Ward, C.L., Omura, S., Kopito, R.R. 1995. Degradation of CFTR by the ubiquitin-Proteasome pathway. *Cell* **83:**121–127

Wine, J.J. 1988. The heterozygote phenotype in cystic fibrosis. *In:* Cellular and Molecular Basis of Cystic Fibrosis, G. Mastella and P.M. Quinton, editor. pp. 33–46. San Francisco Press, CA

Worrell, R.T., Butt, A.G., Cliff, W.H., Frizzell, R.A. 1989. A volume-sensitive chloride conductance in human colonic cell line T84. *Am. J. Physiol.* **256:**C1111–C1119