Rectal Potential Difference and the Functional Expression of CFTR in the Gastrointestinal Epithelia in Cystic Fibrosis Mouse Models

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ABSTRACT: Cystic fibrosis (CF) is an autosomal recessive disease that results from mutations in the CF transmembrane conductance regulator (CFTR) gene. The effect of interventions aimed at correcting the CF electrophysiologic phenotype has been primarily measured using *in vitro* methods in gastrointestinal and respiratory epithelia. A reliable *in vivo* assay of CFTR function would be of great value in the investigation of pharmacologic interventions for CF mouse models. We performed the *in vivo* rectal potential difference (RPD) assay on three different mouse models. We then compared the *in vivo* data with the results obtained using the *in vitro* Ussing chamber method. The results from the *in vitro* method correlated closely with the results acquired using the *in vivo* method and were reproducible. The data suggest that the *in vivo* RPD assay is a reliable assay of functional CFTR expression in CF mouse models. (*Pediatr Res* 63: 73–78, 2008)

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which codes for the cystic fibrosis transmembrane conductance regulator (CFTR) (1–3). CFTR, a member of the adenosine triphosphate binding cassette transporter glycoprotein superfamily, functions as a chloride channel and regulates other ion channels in the apical membrane of certain epithelia. Over a thousand disease-causing mutations have been identified in the CFTR gene. These mutations have been separated into five major subtypes, which include null/decreased production (Class I), improper processing and trafficking (Class II), ion channel dysregulation (Class III), altered channel conduction (Class IV), and improper protein targeting (Class V). The most common mutation in the CFTR gene is $\Delta F508$, a Class II mutation in which the CFTR protein is retained in the endoplasmic reticulum and targeted for degradation (4–6). $\Delta F508$ -CFTR can function as a chloride channel but is unable to reach the apical membrane (7,8).

A number of future therapeutic strategies for CF are focused on correcting or circumventing the mutant CFTR gene, with the hope that this will result in decreased pathology caused by the primary gene defect. A variety of compounds are being investigated as potential treatments in CF, for

instance, 4-phenylbutyrate, trimethylamine oxide (TMAO), and curcumin, as well as flavonoids. 4-Phenylbutyrate, TMAO, and curcumin have been shown to increase $\Delta F508$ -CFTR surface expression in a number of *in vitro* systems (9–11), whereas once at the membrane, flavonoid compounds (genistein, apigenin) and vitamin C have been suggested to activate $\Delta F508$ -CFTR (12–14). In addition, gentamicin, an aminoglycoside antibiotic, has been shown to suppress premature termination codons and restore CFTR function in patients with the W1282X mutation (15). Other interventions, such as gene therapy using viral vectors and stem cell transplant, have also been suggested as potential interventions in CF.

Assessing the effects of the various interventions aimed at correcting the CF electrophysiologic phenotype in affected epithelia is primarily performed using *in vitro* electrophysiologic methods such as examining excised tissue mounted in Ussing chambers. The nasal potential difference (PD) assay is a widely accepted *in vivo* method, which has been used to assess functional expression of CFTR in respiratory epithelia in humans and was pioneered by Knowles *et al.* (16,17) in the 1980s. This method has been adapted by Grubb (18) and now allows for the investigation of many potential interventions for CF in various mouse models of CF. Similarly, a reliable *in vivo* assay of CFTR function in intestinal epithelia could be of great value in the investigation of pharmacologic interventions for CF mouse models. It has been suggested that the rectal potential difference (RPD) assay is a reliable *in vivo* method for assessment of CFTR activity (10, 11, 19). However, there are no reported studies that evaluate the reliability and reproducibility of this method.

METHODS

Mice. Two models of gene-targeted mice for the $\Delta F508$ mutation were used in the experiments. One of the mouse models was bred on a mixed

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Abbreviations: Bl6, Black 6; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CFTR^(-/+), heterozygous for wild type CFTR and mutant CFTR; CFTRinh-172, a specific CFTR channel inhibitor; DCPD, distal colon potential difference; $\Delta F508$, deletion of phenylalanine at the 508 position; ΔPD_{fsk} , mean change in potential difference in response to forskolin; KBR, Krebs's bicarbonate Ringer's solution; PD, potential difference; RPD, rectal potential difference; TMAO, trimethylamine oxide

Figure 1. Representative tracings of *in vivo* DCPD. Intestinal PD was measured using a probing bridge made from PE50 tubing inserted 15 mm in depth, as outlined in the “Methods” section. During the initial period, the PD is measured in a chloride-free Ringer’s solution containing amiloride and barium hydroxide. Subsequently, forskolin is added to the perfusing solution. (A) Representative tracing from a nonCF animal. (B) CF-affected mouse.

genetic background [approximately 25% 129 and 75% Black 6 (B16) (20)], whereas the other $\Delta F508$ mouse model was further backcrossed into a pure B16 background. We refer to these mouse models respectively as the mixed genetic background and pure B16 background $\Delta F508$ mice. A transgenic CFTR^{-/-} knockout (B6.129P2Cfr^{tm1Unc}) was also used in the experiments as a CFTR-null model (21,22). The $\Delta F508$ -CFTR^{+/+} and the CFTR-null mice have severe gastrointestinal manifestations, including bowel obstruction, bowel strictures, and peritonitis. All mice were fed with Harlan Teklad 9F food (Harlan Teklad, Madison, WI) and drinking water that was supplemented with 17.5 g/250 mL of Colyte (Schwarz Pharma, Milwaukee, WI) to improve the survival of the animals (23). All three CF mouse models were bred to obtain wild-type, heterozygote, and CF-affected animals for each model system, were maintained at the Yale University Animal Facility, and were genotyped with standard protocols. All procedures were performed in compliance with relevant laws and institutional guidelines and were approved by the Yale University Institutional Animal Care and Use Committee.

***In vivo* potential difference measurements.** The intestinal PD was measured using a probing bridge made from PE50 tubing inserted into the rectum of the mouse. A 3 mm insertion depth for RPD and a 15 mm insertion depth for “distal colon” PD (DCPD) were used. AgCl₂ voltage electrodes were connected by way of 3% agar bridges made with 3 M KCl. The insertion depths for the rectal and distal colon have been confirmed at the time of sacrifice to ensure proper placement of the tubing. The experimental electrode was connected through the probing bridge, and the reference electrode was connected through a 23-gauge butterfly needle filled with normal saline and placed subcutaneously. A microperfusion pump controlled the flow of perfusate through the probing electrode (0.5 mL/h). A separate length of PE50 tubing was inserted in the rectum and attached to a suction apparatus to minimize distension of the gut. During the procedure, the mice were maintained on a warming pad, and saline drops or artificial tears were used to keep their eyes moist. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally before the procedure. Mice were continually observed throughout the procedure and postprocedure until they were fully recovered.

Table 1a. *In vivo* distal colon potential difference parameters from three CF mouse models

	<i>n</i>	ΔPD_{fsk} (mV)	Baseline PD (mV)	Peak forskolin PD (mV)
Mixed background $\Delta F508$				
Wild type	4	-11.9 ± 1.7	-10.8 ± 1.1	-22.7 ± 0.8
Heterozygous	13	-7.9 ± 1.3	-8.3 ± 0.8	-16.2 ± 1.8
CF*	18	3.5 ± 0.3	-1.4 ± 0.7	2.1 ± 0.7
CFTR-null				
Wild type	6	-13.3 ± 2.1	-9.5 ± 2.1	-22.8 ± 3.5
Heterozygous	12	-6.6 ± 1.6	-7.7 ± 0.7	-16.3 ± 1.6
CF**	11	3.9 ± 0.4	-2.4 ± 1.0	1.5 ± 1.2
Pure B16 background $\Delta F508$				
Wild type	5	-11.9 ± 1.0	-9.7 ± 1.5	-21.6 ± 1.7
Heterozygous	10	-9.5 ± 1.6	-7.8 ± 0.9	-16.5 ± 2.0
CF†	5	3.9 ± 0.6	1.5 ± 1.1	5.4 ± 1.2

Table 1b. *In vitro* distal colon potential difference parameters from three CF mouse models

	<i>n</i>	ΔPD_{fsk} (mV)	Baseline PD (mV)	Peak forskolin PD (mV)
Mixed background $\Delta F508$				
Wild type	4	-12.4 ± 1.9	-8.8 ± 0.5	-20.6 ± 2.3
Heterozygous	17	-10.3 ± 1.9	-7.5 ± 0.8	-17.5 ± 2.6
CF*	10	0.9 ± 0.3	-2.0 ± 0.4	-1.5 ± 0.5
CFTR-null				
Wild type	6	-9.9 ± 1.4	-8.8 ± 1.4	-18.8 ± 1.6
Heterozygous	10	-10.5 ± 1.3	-8.6 ± 1.8	-19.3 ± 1.7
CF**	9	1.1 ± 0.2	-0.4 ± 1.1	0.7 ± 1.2
Pure B16 background $\Delta F508$				
Wild type	5	-8.7 ± 0.9	-6.9 ± 1.0	-15.6 ± 0.5
Heterozygous	10	-9.3 ± 0.9	-5.3 ± 1.0	-14.9 ± 1.6
CF†	5	1.5 ± 0.3	0.7 ± 0.7	2.3 ± 1.0

Values are means $\pm$ SEM.

n, number of tissues; PD, potential difference; ΔPD_{fsk} , change in PD in response to forskolin.

* $p < 0.05$ for CF vs heterozygote and wild-type mice for the mixed background for all parameters using either assay, ** $p < 0.05$ for CF vs heterozygote and wild-type mice for the CFTR null background for all parameters using either assay, † $p < 0.05$ for CF vs heterozygote and wild-type mice for the pure background for all parameters using either assay.

In vitro potential difference measurements. We performed the *in vitro* analysis as previously described by Grubb (18). The rectum and distal colon were removed, cut longitudinally, washed of any intestinal contents, and mounted in the Ussing chamber with an aperture size of 0.04 cm² for rectal tissue and 0.3 cm² for distal colonic tissue (Physiologic Instruments, San Diego, CA). The PD was measured under open-circuit conditions. During all experiments, solutions were continuously gassed with 95% O₂/5% CO₂ and warmed to 37°C. Data were recorded continuously using the Acquire and Analyze software program (Physiologic Instruments, San Diego, CA).

Solutions. The solutions used in this study were a Kreb's bicarbonate Ringer's solution (KBR) and a chloride-free Ringer's solution containing 5 mM barium hydroxide. Amiloride and forskolin were added to the chloride-free Ringer's solution at final concentrations of 10⁻⁴ M and 10⁻⁵ M, respectively. In a subset of experiments, a specific CFTR inhibitor, CFTRinh-172, was added to the final perfusate. All drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and J. T. Baker (Phillipsburg, NJ).

Statistics. The *t* test was used to compare groups for significant differences. A *p* value of less than 0.05 was used to signify statistical significance. All results are reported as the mean ± SEM.

RESULTS

In vivo intestinal potential difference. The *in vivo* intestinal PD was measured for 30 min. Initially, a KBR solution is perfused for a baseline measurement. It is quickly switched to the first perfusate solution that is used in the RPD assay, a chloride-free Ringer's solution containing Ba(OH)₂ and amiloride, as previously described by Fischer *et al.* (10,14), and the transepithelial PD is recorded for 15 min. The addition of amiloride and barium block sodium and potassium currents that could contribute to the PD. Subsequently, forskolin was

added to the perfusate, and the PD was measured for an additional 15 min to activate CFTR chloride current. Data were obtained from two separate regions of the murine intestinal tract, the distal colon and the rectum (Fig. 1). The baseline PD, the mean change in the PD in response to forskolin (Δ PD_{fsk}), and the peak forskolin PD for all CF mouse models were obtained for distal colon (Table 1) and rectum (Table 2).

At baseline, *in vivo* intestinal PD measurements of wild-type and heterozygote mice revealed a modest hyperpolarization, which was similar in magnitude for all three models. Furthermore, in wild-type and heterozygous mice, the addition of forskolin resulted in a further hyperpolarization (a more negative PD measurement). This observation held true in all three models for both the distal colon and rectum (Tables 1 and 2). Interestingly, in both distal colon and rectum, the wild-type mice demonstrated a more negative baseline PD, a more negative peak PD in response to forskolin, and a larger Δ PD_{fsk} than the heterozygous mice (Tables 1 and 2). However, these trends did not reach statistical significance except in the CFTR-null mouse model.

The baseline PD in CF-affected mice was significantly more positive when compared with the heterozygous and wild-type mice. This observation held true for all three CF mouse models regardless of the intestinal region studied (Tables 1

Table 2a. *In vivo* rectal potential difference parameters from three CF mouse models

	<i>n</i>	Δ PD _{fsk} (mV)	Baseline PD (mV)	Peak forskolin PD (mV)
Mixed background Δ F508				
Wild type	4	-8.2 ± 0.9	-7.1 ± 2.1	-15.4 ± 2.4
Heterozygous	18	-7.6 ± 1.0	-6.9 ± 1.4	-13.0 ± 1.7
CF*	10	5.6 ± 2.1	-2.2 ± 1.2	3.4 ± 3.2
CFTR-null				
Wild type	5	-9.1 ± 1.5	-11.0 ± 2.3	-20.3 ± 3.0
Heterozygous	10	-7.3 ± 1.2	-7.9 ± 0.9	-14.7 ± 1.6
CF**	13	3.6 ± 0.6	-0.5 ± 1.4	3.1 ± 1.3
Pure Bl6 background Δ F508				
Wild type	5	-11.3 ± 1.6	-10.9 ± 1.2	-21.2 ± 1.6
Heterozygous	10	-10.4 ± 2.2	-5.3 ± 1.5	-16.8 ± 2.1
CF†	5	3.4 ± 1.1	2.6 ± 1.3	6.0 ± 1.9

Table 2b. *In vitro* rectal potential difference parameters from three CF mouse models

	<i>n</i>	Δ PD _{fsk} (mV)	Baseline PD (mV)	Peak forskolin PD (mV)
Mixed background Δ F508				
Wild type	4	-11.1 ± 3.9	-6.7 ± 0.5	-17.9 ± 3.4
Heterozygous	10	-8.4 ± 2.3	-6.9 ± 0.8	-15.3 ± 2.3
CF*	10	0.7 ± 0.5	-2.9 ± 0.8	-2.3 ± 1.1
CFTR-null				
Wild type	6	-7.1 ± 2.5	-6.2 ± 0.8	-12.2 ± 2.7
Heterozygous	10	-11.5 ± 1.3	-8.3 ± 1.8	-19.8 ± 1.7
CF**	7	1.8 ± 0.8	-2.2 ± 1.2	-0.4 ± 1.5
Pure Bl6 background Δ F508				
Wild type	5	-7.0 ± 1.1	-6.7 ± 0.8	-13.1 ± 1.7
Heterozygous	10	-5.3 ± 1.2	-3.4 ± 1.3	-8.7 ± 1.3
CF†	5	1.9 ± 0.5	0.07 ± 1.0	2.7 ± 1.0

Values are means ± SEM.

n, number of tissues; PD, potential difference; Δ PD_{fsk}, change in PD in response to forskolin.

* *p* < 0.05 for CF vs heterozygote and wild-type mice for the mixed background for all parameters using either assay, ** *p* < 0.05 for CF vs heterozygote and wild-type mice for the CFTR null background for all parameters using either assay, † *p* < 0.05 for CF vs heterozygote and wild-type mice for the pure background for all parameters using either assay.

and 2). In addition, whereas all of the wild-type and heterozygous mice demonstrated a hyperpolarization of the PD in response to forskolin, the CF-affected mice demonstrated a depolarization of the PD in response to forskolin (Tables 1 and 2). These differences are similar to previously reported *in vivo* findings and are consistent with the CF phenotype (10).

To demonstrate that the forskolin stimulated current was caused by CFTR activity, 20 μ M of CFTRinh-172, a specific CFTR inhibitor, was added to the perfusate solution after forskolin stimulation in a subset of experiments (24). The addition of the blocker significantly inhibited the hyperpolarization that was observed in the wild-type and heterozygote animals in all three mouse models ($n = 16$). These findings suggest that the majority of the current activated by forskolin

Figure 2. CFTRinh-172 inhibits the forskolin stimulated hyperpolarization observed in the *in vivo* RPD/DCPD assay in all three CF animal models. Twenty micromolar of CFTRinh-172 was added to the chloride-free Ringer's solution containing amiloride, barium hydroxide, and forskolin. Mixed background ΔF508-CFTR colony ($n = 6$); CFTR null colony ($n = 5$); pure B16 background ΔF508-CFTR colony ($n = 5$).

was caused by CFTR activation. No effect was observed in the CF-affected animals ($n = 6$) (Fig. 2).

***In vivo* ΔPD_{fsk} is a reproducible measurement when assayed repeatedly on a single animal.** To test the reproducibility of the *in vivo* methods, we repeated three *in vivo* DCPD assays on five heterozygous and six CF-affected mice at 2 wk to monthly intervals. Although there is some variability in the absolute values, the ΔPD_{fsk} remained similar for all of three experiments for each animal (Fig. 3). For instance, the ΔPD_{fsk} for one of the heterozygous mice was -10.7 , -11.2 , and -9.6 mV, respectively, and for one of the CFTR-null mice was 3.3 , 2.0 , and 2.5 mV, as noted in Fig. 3.

***In Vivo* Intestinal Potential Difference versus *In Vitro* Intestinal Potential Difference.** Next, to test whether the *in vivo* PD assay results correlated with those observed in an *in vitro* assay, we measured the *in vitro* intestinal PD using the Ussing chamber. Before the *in vitro* assay, *in vivo* PD measurements were obtained on each animal. Immediately after the *in vivo* assay, the animals were killed, and the *in vitro* assay was performed. Data were again obtained from two separate regions of the murine intestinal tract, the distal colon and the rectum, after a 20-min equilibration period in KBR. The same protocol and solutions were used in the Ussing chamber as in the *in vivo* PD method. The mean baseline RPD and DCPD were similar in all three mouse models regardless of whether the Ussing chamber or the RPD method was used to obtain measurements (Figs. 4 and 5).

For all three CF mouse models, the forskolin response in both the distal colon and rectum appeared similar in magnitude when using either *in vivo* or *in vitro* methods to measure the ΔPD_{fsk} (Figs. 4 and 5), and the inhibition of the forskolin response by CFTRinh-172 was also similar. There was only one significant difference observed between *in vivo* and *in vitro* measurements, and it was observed in the CF-affected

Figure 3. *In vivo* PD measurements in CF-affected and normal mice are reproducible. Repeated DCPD measurements were obtained in three separate experiments on one heterozygote (CFTR null mouse) and on one homozygote CFTR null mouse. (A) PD measurements for a heterozygous (CFTR-null) mouse are graphed over time: trial #1 (◆), trial #2 (■), trial #3 (▲), and mean values (○) for the three experiments. Horizontal black bar represents a time length of 15 min. Black arrow denotes change to forskolin containing solution. (B) ΔPD_{fsk} for each trial depicted in A: trial #1 (□), trial #2 (■), trial #3 (▤), and the mean (▨) ΔPD_{fsk} for the three experiments. (C) The average ΔPD_{fsk} for three repeated RPD assays on five heterozygote mice. (D) PD measurements for a homozygous (CFTR-null) mouse are graphed over time: trial #1 (◆), trial #2 (■), trial #3 (▲), and the mean values (○) for the three experiments. Horizontal black bar represents a time length of 15 min. Black arrow denotes change to forskolin containing solution. (E) ΔPD_{fsk} for each trial is depicted in D: trial #1 (□), trial #2 (■), trial #3 (▤), and the mean (▨) ΔPD_{fsk} for the three experiments. (F) The average ΔPD_{fsk} for three repeated RPD assays on six CF-affected mice. In all panels, error bars represent the SEM.

Figure 4. Comparison of the observed change in PD in response to 10 μ M forskolin (ΔPD_{fsk}) in wild-type ($\square$), heterozygous ($\▤$), and CF-affected mice ($\blacksquare$) in three CF mouse models. *In vitro* results are represented on the left side of each panel and *in vivo* results on the right side. $n = 5$ for all groups (wild type, heterozygote, CF affected) and both tissues (rectum and distal colon). $*p < 0.05$ between *in vivo* and *in vitro* results for distal colon in CF-affected groups only. (A) $\Delta F508$ -CFTR mixed background mouse model. (B) CFTR-null CF mouse model. (C) $\Delta F508$ -CFTR pure B16 background mouse model.

mice. In the CF mice, there was a greater depolarization in the *in vivo* distal colon (ΔPD_{fsk}) analysis compared with the *in vitro* results. This was true for all three CF mouse models. The distal colon ΔPD_{fsk} measurements were 3.8 ± 0.4 mV *versus* 0.9 ± 0.3 mV for mixed genetic background $\Delta F508$ mouse model, 3.7 ± 0.3 mV *versus* 1.1 ± 0.2 mV for CFTR-null model, and 3.9 ± 0.6 mV *versus* 1.5 ± 0.3 for pure B16 background $\Delta F508$ mouse model. A similar trend was also observed for the rectal PD of the CF mice; however, it was not of statistical significance. Last, a significant inhibition of the forskolin response was observed with the addition of CFTRinh-172 in both the wild-type and heterozygous tissues, similar to what observed in the *in vivo* assay ($n = 9$, data not shown). Once again, there was no effect of CFTRinh-172 on the CF-affected tissue ($n = 5$, data not shown).

DISCUSSION

The *in vivo* rectal PD assay appears to be a reliable and reproducible assay of functional CFTR expression in CF mouse models. In our study, the *in vivo* PD measurements

Figure 5. Comparison of the mean PD measured at minute intervals using the *in vitro* assay (closed symbols) or the *in vivo* (open symbols) assay. CF results are represented as circles, wild type as squares, and heterozygote as triangles. Horizontal black bar represents a time length of 15 min. Black arrow denotes change to forskolin containing solution. (A and B) Traces for the mixed background $\Delta F508$ mice (distal colon A, rectum B). (C and D) Traces for the CFTR-null mice (distal colon C, rectum D). (E and F) Traces from the pure B16 background $\Delta F508$ mice (distal colon E, rectum F).

correlated very closely with the PD measurements acquired using the *in vitro* method, suggesting that the *in vivo* PD assay may be a very useful alternative method for studying ion transport in CF-affected animals if serial measurements are required as part of a study design. Although the results of the two methods are nearly identical, there were interesting subtle differences when comparing techniques. Of note, in CF-affected animals, the degree of depolarization observed in response to forskolin appeared to be greater when using the *in vivo* assay than the *in vitro* assay.

In addition, in the *in vivo* PD assay, wild-type mice demonstrated a larger ΔPD_{fsk} , a more negative baseline PD, and a more negative peak PD in response to forskolin compared with their heterozygote counterparts, suggesting that there is a greater degree of functional CFTR expression in wild-type than in heterozygous mice. This trend was noted for all three CF mouse models studied, in both the distal colon and rectum. Our *in vivo* data correlate with the observations of Gabriel *et al.* (25). They reported that heterozygote CFTR^(-/+) mice were more resistant to secretory diarrhea after exposure to cholera toxin when compared with wild-type animals. They postulated that this heterozygote advantage was caused by less CFTR expression in the CFTR heterozygous mice (26). Interestingly, the PD measurements from the same mice using the *in vitro* method did not show statistically significant differences between the wild-type and heterozygous groups, which is similar to the findings of Cuthbert *et al.* (26) and is consistent the initial characterization of intestinal transport by *in vitro* methods in the $\Delta F508$ mixed genetic background mouse model (19). We speculate that the subtle differences

that are detected using the *in vivo* method result from the fact that the tissue is *in situ* and is subject to the endogenous neural-humoral supply of the gut. This is distinctly different from the removal and mounting of the same tissue in an artificial environment to maintain tissue viability.

Both methods of studying intestinal transport have their limitations. For example, serial measurements of CFTR functional expression are not possible when using the *in vitro* method. On the other hand, it is impossible to control the internal and external environments affecting the tissue when using the *in vivo* method. However, both methods used in concert can yield important details regarding the ion transport properties of the intestinal epithelia. The power of the *in vivo* method is its ability to measure CFTR functional expression before and after interventions aimed at correcting the CF electrophysiologic phenotype.

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