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Ileal smooth muscle dysfunction and remodeling in cystic fibrosis

P.-A. Risse, L. Kachmar, O. S. Matusovsky, M. Novali, M. Gil FR, Javeshghani S, Keary R, Haston CK, Michoud M-C, Martin JG, Lauzon A-M. Ileal smooth muscle dysfunction and remodeling in cystic fibrosis. Am J Physiol Gastrointest Liver Physiol 303: G1–G8, 2012. First published April 26, 2012; doi:10.1152/ajpgi.00356.2011.—Patients with cystic fibrosis (CF) often suffer from gastrointestinal cramps and intestinal obstruction. The CF transmembrane conductance regulator (CFTR) channel has been shown to be expressed in vascular and airway smooth muscle (SM). We hypothesized that the absence of CFTR expression alters the gastrointestinal SM function and that these alterations may show strain-related differences in the mouse. The aim of this study was to measure the contractile properties of the ileal SM in two CF mouse models. CFTR−/− and CFTR+/− mice were studied on BALB/cJ and C57BL/6J backgrounds. Responsiveness of ileal strips to electrical field stimulation (EFS), methacholine (MCh), and isoproterenol was measured. The mass and the cell density of SM layers were measured morphometrically. Finally, the maximal velocity of shortening (V_{max}) and the expression of the fast (+) insert myosin isoform were measured in the C57BL/6J ileum. Ileal hyperreactivity was observed in response to EFS and MCh in CFTR−/− compared with CFTR+/+ mice in C57BL/6J background. This latter observation was not reproduced by acute inhibition of CFTR with CFTRinh172. BALB/cJ CFTR−/− mice exhibited a significant increase of SM mass with a lower density of cells compared with control and CFTR+/+ mice in C57BL/6J background. Thus we investigated the response to electric field stimulation (EFS) the reactivity and sensitivity in response to muscarinic (MCh) (17, 29, 32). In addition to the respiratory problems, CF patients also frequently suffer from abdominal cramps, esophageal and intestinal dysmotility, distal intestinal obstruction syndrome, and gastrointestinal reflux (6, 16, 17, 21). They also commonly suffer from bradygastria (3). These disorders are thought to be secondary to the thick mucus lining the gastrointestinal tract, but dysfunctional intestinal SM could also contribute significantly to these problems.

The expression and function of CFTR in SM has previously been studied in vascular and airway tissues (11, 20, 24, 25). Activation of the CFTR channel by the vasoactive intestinal peptide induces relaxation of precontracted rat vascular SM preparations. In addition, vascular SM strips from CFTR−/− mice constrict more than wild-type mice, and their relaxation in the presence of CFTR activators is impaired. The CFTR channel is also functional in rat tracheal SM cells (31) and human airways SM cells (20). Human CF airway SM cells are also known to contract more to IL-8 than non-CF cells (11).

Based on these observations, we reasoned that the SM of the small intestine might be dysfunctional in the absence of functional CFTR. Previous work also reported mouse strain differences in the pulmonary gene expression of CFTR−/− (12).

The aim of this study was to compare the functional and morphological properties of the ileal SM of CFTR−/− and CFTR+/+ mice from C57BL/6J and BALB/cJ background. Thus we investigated the response to electric field stimulation (EFS), the reactivity and sensitivity in response to muscarinic and β_{2}-adrenergic receptor agonist, the velocity of shortening, and the morphology of ileal SM strips.

MATERIAL AND METHODS

Animals and tissue preparation. All experimental protocols involving animals were approved by the McGill University Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care. Utilizing the CF murine model CFTR<sup>−/−</sup>, congenic C57BL/6J and BALB/cJ CFTR<sup>−/−</sup> mice were maintained in a breeding colony at the Meakins-Christie Laboratories of McGill University as previously described (12, 13). The CFTR<sup>−/−</sup> mice were intercrossed to produce CFTR<sup>+/−</sup>, CFTR<sup>+/−</sup>, and CFTR<sup>−/−</sup> mice, which were identified by genotyping as previously described (12). To circumvent premature death of CFTR<sup>−/−</sup> mice as a result of intestinal disease, mice were fed standard chow and received PegLyte [polypehylene glycol (17.8 mM) and electrolytes; Pharmascience, Montreal, PQ, Canada] in their drinking water. Mice were euthanized with pentobarbital sodium at 12 wk of age. A 1-cm-long piece of ileum was cleaned and dissected in ice-cold Krebs-Henseleit (K-H; in mM: 118 NaCl, 4.51 KCl, 2.46 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25.5 NaHCO<sub>3</sub>, 10 glucose, and 2.5 CaCl<sub>2</sub>).
Functional studies of the ileal longitudinal muscle. Ileal SM strips were mounted in a horizontal organ bath containing a K-H solution maintained at 37°C. One end of the strip was attached to a fixed hook and the other end to a dual-mode force transducer and high-speed length controller system (model 300B-LR, Aurora Scientific). A preload of 0.5 g was applied to the muscle strip. The muscle was equilibrated for 1 h at 37°C in K-H solution bubbled with 95% O2-5% CO2 mixture. To determine the optimal length (l0), the muscle strip underwent repeated EFS at 50 V, 100 Hz, pulse width of 2 ms, 5-s duration, at 5-min intervals. l0 was found by increasing the muscle length after each EFS until the active force reached a maximal stable response. Peak isometric force (Fmax) was measured in response to EFS (same parameters as above) and was repeated in the presence of increasing concentrations of isoproterenol (10^-10-10^-6 M, Sigma) (Fig. 1A). The tissue reactivity and sensitivity in response to contractile and relaxant agonists were assessed by measuring isometric force changes in response to cumulative concentrations of methacholine (MCh) (10^-10-10^-4 M, Sigma) followed by cumulative concentrations of isoproterenol (10^-10-10^-6 M) (Fig. 1B). To evaluate the potency of the agonists, the maximum response (Emax), the concentration for half-maximal response (EC50), and the pD2 (the negative logarithm of the EC50) values were calculated from the concentration-response curve obtained in each experiment.

Fig. 1. Representative isometric force traces of mouse ileal smooth muscle (SM) when stimulated by electric field stimulation (EFS) every 5 min in the presence of increasing concentrations of isoproterenol (10^-10-10^-6; A); cumulative addition of methacholine (MCh) (10^-8-10^-4 M) followed by cumulative addition of isoproterenol (10^-10-10^-6 M), added every 2 min (B); maximal concentration of MCh (10^-4 M) followed by 15 min of washout, followed by a 30-min incubation in Krebs-Henseleit solution containing 0.1% of DMSO (C) or 10^-4 M CFTRinh172 (D), followed by a cumulative addition of MCh (10^-8-10^-4 M) and isoproterenol (10^-10-10^-6 M).
To directly evaluate the role of the CFTR channel in SM function, the contractile response to MCh was measured in the presence of CFTRinh172 (Tocris, Minneapolis, MN). Briefly, at the end of the equilibration period, a response to 10^{-4} M MCh was measured. Then, after 15 min of washout, tissues were preincubated with vehicle (DMSO 0.1%) or with CFTRinh172 at 10^{-4} M (31) (Fig. 1D) for 30 min before cumulative additions of MCh and isoproterenol.

The maximal velocity of muscle shortening (Vmax) was evaluated by constructing force-velocity curves using the quick release technique as follows. After equilibration, Fmax was obtained in response to EFS (50 V, 100 Hz, pulse width of 2 ms, 5-s duration). At 2-min intervals, EFS was triggered to stimulate the muscle isometrically. At 1.5 s after the onset of the stimulus, the muscle length was suddenly dropped to a selected afterload tension. The rate of shortening was measured at 100 ms after the release. By repeating these measurements at several afterloads in random order, a complete force-velocity curve was constructed, and Vmax was extrapolated by fitting the data to the Hill equation (15). The force-velocity curves were constructed only for the C57BL/6J mice because in BALB/cJ mice the clamping of the force transducer was below the detection level of the force transducer.

Morphometric analysis of the ileal SM mass. Pieces of the longitudinal and circular ileum were fixed in formalin for 12 h and subsequently embedded in paraffin. Sections of 5-μm thickness were then cut using a microtome (model 2040 AUTOCUT). The muscle area was estimated by measuring SM α-actin (α-SMA) immunoreactive regions as previously described (30). The sections were incubated in the dark with the mouse on mouse kit (M.O.M., Vector Laboratories) Ig blocking reagent for 1 h and then stained with a mouse monoclonal antibody to α-SMA (clone 1A4; Sigma, Saint-Louis, MO) and a biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories). Negative controls were run using the same concentration of isotype control IgG (R&D systems) in place of primary antibody. The signal was detected with Vector Red (Vector Laboratories) and then stained with a mouse polyclonal antibody to α-SM muscle heavy chain (SMMHC) (clone SMA, 1:400; Sigma, Saint-Louis, MO) and a biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories). Negative controls were run using the same concentration of isotype control IgG (R&D systems) in place of primary antibody. The signal was detected with Vector Red (Vector Laboratories) and then stained with a mouse monoclonal antibody to total SM myosin, the pD2, the Vmax, and the protein expression were tested using Western blot analysis.

Fig. 2. EFS-induced ileal longitudinal SM contraction. Resting tone of the ileal longitudinal SM from CFTR^{−/−} and CFTR^{+/+} was similar in the two strains of mice (Fig. 2A). Fmax in response to EFS in C57BL/6J mice, whereas no difference was observed in BALB/cJ (B). No difference was observed in the inhibition of EFS-induced contraction by isoproterenol in C57BL/6J (C), whereas tissues from BALB/cJ CFTR^{−/−} mice exhibited a greater relaxation than the CFTR^{+/+} (D). N = 6 per group. *Significant difference (P ≤ 0.05).
to EFS was measured in the absence and presence of increasing concentrations of isoproterenol. The C57BL/6J CFTR−/− mice developed a significantly greater Fmax compared with the CFTR+/+ mice (Fmax = 33.1 ± 4.2 vs. 16.5 ± 0.8 mN, n = 4–6; P ≤ 0.05; Fig. 2B), whereas in BALB/cJ mice no significant difference was observed between CFTR−/− and CFTR+/+ mice (Fmax = 16.9 ± 4.2 vs. 11.3 ± 1.5 mN, n = 3–5). The myorelaxant effect of isoproterenol on EFS-induced contractions was not significantly different between CFTR−/− and CFTR+/+ animals in the C57BL/6J; however, the relaxation effect was significantly greater in the CFTR−/− BALB/cJ mice compared with the CFTR+/+ mice (Fig. 2, C and D).

Longitudinal ileal SM responses to contractile and relaxant agonists. Cumulative concentration-response curves to MCh were constructed (Fig. 3, A and B). The contractile response to MCh was stronger in the C57BL/6J compared with the BALB/cJ strain. In the C57BL/6J strain, the SM contraction induced by MCh was significantly increased in CFTR−/− compared with the CFTR+/+ mice (ΔFmax/10 M MCh = +42.2 ± 10.4 vs. +20.0 ± 1.62 mN, n = 3–4; P ≤ 0.05; Fig. 3A); whereas no significant difference was observed in the BALB/cJ strain (n = 4–5; Fig. 3B). By contrast, loss of CFTR expression did not affect isoproterenol-induced relaxation (Fig. 3, C and D) or the tissue sensitivity to MCh and isoproterenol (see Table 1 for pD2) in both strains of mice.

Effect of CFTRinh172 on the longitudinal ileal SM response to contractile and relaxant agonists in C57BL/6J mice. In the C57BL/6J mice, CFTRinh172 reduced the response to 10−6 M MCh (37.2 ± 11.8 vs. 89.4 ± 13.7% of the first MCh; P < 0.05, n = 6; Fig. 4A) and 10−5 M MCh (91.8 ± 4.5 vs. 129.7 ± 13.0% of the first MCh; P < 0.05, n = 6) but did not modify the potency of MCh (pD2 = 6.3 ± 0.1 in DMSO group and 5.8 ± 0.2 in CFTRinh172 group; n = 6). In contrast, CFTRinh172 did not change the relaxation induced by isoproterenol (Fig. 4B).

Morphometric analysis of the ileum SM mass. The mass of the circular and longitudinal SM layers was significantly greater in the BALB/cJ CFTR−/− animals than in the CFTR+/+ mice (P ≤ 0.05, n = 4–5; Fig. 5, A and B). This increase of SM mass was accompanied by a significant decrease of density of cells in all SM layers of CFTR−/− compared with CFTR+/+ (P ≤ 0.05, n = 4–5; Fig. 5, C and D). In contrast, no significant differences in mass or cell density were found in any layer of the ileal SM between the CFTR−/− and the CFTR+/+ mice in the C57BL/6J strain. Representative examples of tissue sections show SM cells (red

Table 1. Values of the negative logarithm of the EC50 for MCh and isoproterenol for the longitudinal ileal smooth muscle strips

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<th>C57BL/6J</th>
<th>BALB/cJ</th>
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<tr>
<td>CFTR+/+</td>
<td>6.45 ± 0.07</td>
<td>6.43 ± 0.17</td>
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<tr>
<td>CFTR−/−</td>
<td>6.84 ± 0.18</td>
<td>6.66 ± 0.13</td>
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<tr>
<td>Isoproterenol</td>
<td>7.08 ± 0.09</td>
<td>6.51 ± 0.18</td>
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<tr>
<td>CFTR+/+</td>
<td>6.96 ± 0.08</td>
<td>6.48 ± 0.15</td>
</tr>
<tr>
<td>CFTR−/−</td>
<td>6.66 ± 0.13</td>
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Values of the negative logarithm of the EC50 for methacholine (MCh) and isoproterenol for the longitudinal ileal smooth muscle strips from CFTR+/+ and CFTR−/− mice of C57BL/6J and BALB/cJ strain. The lack of CFTR expression did not affect the tissue sensitivity to MCh and isoproterenol in both strains of mice. N = 3–5 mice/group.
staining) within the circular (cir) and the longitudinal (lg) layers (Fig. 5E).

**Force-velocity measurements.** Force-velocity curves were obtained for the C57BL/6 mice (see representative example in Fig. 6A). The loss of CFTR expression induced a significant change of longitudinal SM tone. Incubation with 10^{-4} M of CFTRinh172 reduced the SM response to MCh at the concentration of 10^{-3} and 10^{-4} M (A). CFTRinh172 did not affect the isoproterenol-induced relaxation (B). N = 5 mice/group. *Significant difference (P ≤ 0.05).

**Western blot analysis.** The content in total SMMHC and the fast (+)insert SMMHC isoform present in the ileum of C57BL/6J CFTR^{+/+} and CFTR^{-/-} mice was determined at the protein level by Western blot analysis (n = 5–6; Fig. 7). A significantly lower expression of the total SMMHC was observed in CFTR^{-/-} compared with CFTR^{+/+} mice (0.96 ± 0.06 vs. 1.26 ± 0.08; P = 0.05; R2 > 0.94; Fig. 6B).

**DISCUSSION**

The present study provides evidence that the ileal SM function and morphology are affected in murine models of CF. Significant strain-related differences were observed in the responsiveness of the muscle to EFS and to MCh. CFTR^{-/-} mice of the C57BL/6J background had more reactive muscle than the CFTR^{+/+}. This phenomenon was not observed when a CFTR channel blocker is used. There were no differences in MCh-induced and EFS-induced contraction between the CFTR^{-/-} and CFTR^{+/+} in the BALB/C mice background. However, the relaxant effect of isoproterenol was greater in the EFS-stimulated ileum of CFTR^{-/-} mice, but this difference was not reproduced in response to MCh. Despite these findings, the BALB/C mice had an increase in ilial muscle mass. The fast (+)insert SMMHC isoform expression was examined in the C57BL/6J mice and was found to be increased with respect to the total SMMHC content in the CFTR^{-/-} mice. Concomitantly, Vmax of the ilial muscle was also increased in the CFTR^{-/-} mice compared with the CFTR^{+/+} mice. These findings support the involvement of the intestinal muscle in these models of CF and suggest the possibility that SM dysfunction participates in the intestinal pathophysiology.

CF patients are known to suffer from abdominal cramps and intestinal obstruction. Both the ileum and proximal colon are affected in these patients (26). However, the possibility that the contractility of the gastrointestinal muscle might be adversely affected by CFTR deficiency has not been considered. In the present study, we observed an increase in Vmax of the longitudinal SM strips of the ileum from C57BL/6J CFTR^{-/-} mice by comparison with CFTR^{+/+} in response to EFS. Moreover, the isometric force developed in response to exogenous MCh was increased, indicating that this altered contractility is based on postjunctional mechanisms. In addition, the unaltered sensitivity to MCh (unchanged pD2) suggests a phenomenon that is not caused by altered muscarinic receptor function. The hyperresponsiveness observed could be calcium dependent and/or independent, since both of these mechanisms have been described to be implicated in the altered response of human airway SM from CF patients (20). Cholinergic hyperresponsiveness has been reported to affect, with high prevalence, the ciliary muscle and airway SM in subjects with CF (7), although the two abnormalities may not be closely related (8).

No significant alterations of the responsiveness or sensitivity of the ileal SM to the β2-agonist isoproterenol were observed in the C57BL/6J mice. In contrast, although the effect of isoproterenol was weak on EFS-stimulated ileal muscle of BALB/C CFTR^{-/-} mice, it was more pronounced in the ilium of CFTR^{-/-} mice. The lack of differences between BALB/C CFTR^{+/+} and CFTR^{-/-} in response to isoproterenol in MCh-precontracted muscle suggests that effects on cholinergic neurotransmission account for the findings rather than an effect on SM itself (34). Nevertheless, taken together, these findings suggest that the second messenger pathways mediating ileal muscle relaxation are not directly affected by the lack of CFTR expression in the ileal SM. Indeed, the chloride-
dependent relaxation induced by CFTR activators in the vascular and tracheal SM does not appear to be an important component in the response induced by the $\beta_2$ agonist either (25, 31).

The dynamic mechanical properties were only explored in C57BL/6J because the Fmax of the BALB/cJ strain was quite low, and so the clamping at very low loads to build the force-velocity curves was below the detection level of the force transducer. We observed a greater Vmax for the C57BL/6J CFTR$^{+/+}$ mice intestinal SM in response to cholinergic stimulation compared with the C57BL/6J CFTR$^{+/+}$ mice. This finding may be explained in part by a relative increase in fast (I) insert SMMHC isoform to total SMMHC. The molecular basis of these changes is uncertain, but the overexpression of CFTR in the rat lung has been shown to increase expression of total SMMHC in the lung in utero before the confounding effects of inflammation and infection (5).

However, the fact that inhibiting the CFTR channel function with a chemical inhibitor was not able to reproduce the hyperresponsiveness observed in vivo suggests that some or all of the changes observed require chronic deficiency of CFTR or are secondary to the alterations in intestinal func-

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**Fig. 5.** Morphometric analysis of the longitudinal and circular ileal SM. The lack of CFTR expression induced an increase of the SM mass and a parallel decrease of the cell density in the circular (A and C) and longitudinal (B and D) SM layers of the BALB/cJ strain, whereas no difference was observed for C57BL/6J. CFTR$^{+/+}$, open bars; CFTR$^{+/−}$, solid bars, n = 4–5 mice/group. *Significant difference (P < 0.05). E: representative examples of tissue sections show SM cells (red staining) within the circular (cir) and the longitudinal (lg) layers. 
tion or milieu. The effect of parasitic infection on the intestinal muscular function has been examined in guinea pigs infected intraperitoneally with Toxocara canis. Such infection induced longitudinal SM ileum hyperreactivity and hypersensitivity in response to histamine (28). Infection of mice with T. spiralis also resulted in hyperreactivity of the longitudinal muscle layer associated with a greater degree of shortening and hypersensitivity in response to MCh (1). Whether inflammation associated with CFTR deficiency in intestinal muscle (22) could account for the changes requires further exploration. Nonetheless, it remains difficult to reconcile the observed enhanced muscle mechanics with the slower intestinal transit time in mice with CFTR deficiency in the C57 background (9, 10).

Although C57BL/6J CFTR+/+ mice exhibited a hyperreactive ileal SM, there were no histological changes in the ileum. In contrast, the normoresponsive intestinal muscle from BALB/cJ CFTR+/+ mice was characterized by an increased SM mass that was attributable to hypertrophy. This finding supported the observation of an increase of muscularis externa thickness in BALB/cJ CFTR-/- mice (4) and the recent observations reported from the CFTR-/- piglet ileum (19, 27) that exhibited a distal intestinal obstruction syndrome located around the ileo-cecal junction, which was associated with an increased small intestinal SM layer thickness, including the duodenum and the spiral colon. Although attributed to hypertrophy, the investigators did not identify the mechanism of increase in mass. Our data demonstrate that the remodeling process of the SM in BALB/CJ was exclusively caused by hypertrophy. The CFTR-/- porcine model demonstrated that SM remodeling was present in newborn CFTR-/- piglets in the absence of inflammation. Additionally, morphological changes were observed 12 h after birth, indicating that remodeling occurred during fetal life (19). By comparison, in CF patients, the gastrointestinal manifestation of the disease appears only in early life. In 15% of cases, the first symptom after birth is meconium ileus. Distal intestinal obstructive syndrome may begin a few months after birth and become more disabling with age. Interestingly, there is also an increase of the SM mass in airways of CF patients attributable to hyperplasia without hypertrophy in adults (14) and to both hyperplasia and hypertrophy in the airways of children (23).

In conclusion, chronic CFTR deficiency in the mouse leads to functional and structural changes in intestinal SM that are significantly dependent on the background strain, supporting the importance of modifier genes in its phenotypic expression. Ileal muscle from the C57BL/6J mouse has altered contractile properties and myosin isoform expression but normal SM morphology, whereas the BALB/CJ has preserved contractile function but has hypertrophic cells. The basis for these strain differences is not currently known. These findings raise the
question of the role of altered SM function in the gastrointestinal syndrome associated with CF.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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