Failure of cholinergic stimulation to induce a secretory response from the rectal mucosa in cystic fibrosis

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Abstract

The secretory response to cholinergic stimulation was investigated in rectal biopsy specimens from children with cystic fibrosis and a control group using a modified Ussing chamber technique. Acetylcholine (10^{-3} mol/l) increased the short circuit current in 12 control specimens by mean (SEM) 83.0 (16.4) $\mu\text{A}/\text{cm}^2$, but samples from five children with cystic fibrosis failed to exhibit such a response (-1.4 (3.2) $\mu\text{A}/\text{cm}^2$). Amiloride (10^{-4} mol/l), which will inhibit electrogenic sodium absorption in viable tissues, caused similar reductions in the short circuit current of both control and cystic fibrosis tissues (control = -37.7 (7.7) $\mu\text{A}/\text{cm}^2$; cystic fibrosis = -44.0 (9.3) $\mu\text{A}/\text{cm}^2$). Thus, the failure of chloride secretion observed in the small intestine also exists in the rectal mucosa. This observation could be used both to aid diagnosis and to study the basic defect.

Cystic fibrosis is a single gene defect characterised by failure of the mechanism regulating chloride permeability in epithelia.¹ A chloride permeability step is an integral part of the chloride secretory process of the intestine² and the involvement of this tissue in cystic fibrosis has recently been shown by its inability to respond to secretagogue challenge.³⁻⁶ These studies were carried out using tissue from the small intestine, which is relatively inaccessible. The rectal mucosa is more readily available for study and is known to possess mechanisms for the absorption and secretion of electrolytes.⁷ In vivo observations have indicated that in cystic fibrosis the rectum fails to generate a rise in transmural potential difference in response to cyclic adenosine monophosphate-mediated secretagogues.⁸ This study investigates the response of the rectal mucosa to cholinergic stimulation using biopsy specimens from control subjects and children with cystic fibrosis.

Methods

PATIENTS

Tissues from five children with cystic fibrosis (mean age 4.7 years, range 0.1-11.0 years), whose diagnosis had been confirmed by at least two consecutive abnormal sweat tests, were studied. Three children underwent investigation for rectal prolapse that had occurred in spite of the use of pancreatic enzyme supplementation to control steatorrhoea. The remaining two children with cystic fibrosis underwent rectal biopsy to exclude Hirschsprung's disease. The control

group consisted of 12 children (mean age 5.4 years, range 0.1-14.5 years) with normal sweat test results who underwent investigation for meconium ileus, meconium plug syndrome, the differential diagnosis of Hirschsprung's disease, or to exclude inflammatory bowel disease. Patients in both the cystic fibrosis and control groups receiving laxatives were asked to stop these 12 hours before biopsy. No child was receiving parasympathomimetics. The most frequently used medication in control subjects with constipation was the osmotic agent lactulose; one patient in the cystic fibrosis group was also receiving this. Patients undergoing sigmoidoscopy before biopsy were also given a stimulant laxative, sodium picosulphate. Four of the cystic fibrosis patients received this preparation as did 10 of the 12 control subjects. The study was approved by the local ethical committee.

EXPERIMENTAL PROCEDURE

Rectal biopsy specimens, taken 10-20 cm from the anal margin, were obtained using a standard double port paediatric Crosby capsule. This procedure is readily performed without sedation and provides a greater area of undamaged tissue than standard biopsy forceps. After orientation, tissues were mounted as a sheet (exposed area 3 mm²) in a modified Ussing chamber, incubated at 37°C in Krebs bicarbonate saline containing 10 mmol/l glucose, and gassed with 95% O₂/5% CO₂. The potential difference was measured using salt bridge electrodes connected via calomel half cells to a differential input electrometer. Current was applied across the tissue using Ag/AgCl electrodes which made contact with mucosal and serosal solutions via wide bore salt bridges. Tissue resistance was calculated from the change in potential difference induced by a 10 μA current pulse of approximately five seconds duration and corrected for the resistance of the bathing solution, which was determined in a similar manner in the absence of the tissue. The short circuit current generated by the tissue, a reflection of net electrogenic ion transport, was calculated from potential difference and resistance measurements using Ohm's law. After setting up, the tissue was allowed to stabilise for 20 minutes after which time potential difference and resistance were determined at one minute intervals. For each test substance, readings were taken for five minutes before and 10 minutes after its addition. The response of the tissue to cholinergic stimulation was taken as the difference between the maximum current generated in the presence of acetylcholine (10^{-3} mol/l in the serosal solution) and the value immediately

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Figure 1: Basal electrical activity of 12 control (○) and 5 cystic fibrosis (●) rectal biopsy specimens. Each point represents an individual tissue with the bars indicating mean (SEM) values. A significant ($p < 0.05$) difference between control and cystic fibrosis values is denoted by *. Pd=potential difference; SCC=short circuit current; R=tissue resistance.

before its addition, while the effect of amiloride (10^{-4} mol/l) was taken as the difference between the current immediately before and five minutes after its addition to the mucosal solution. Histological examination of the tissues showed no abnormalities.

EXPERIMENTS WITH RABBIT RECTAL MUCOSA

To assess the influence of a small tissue area on the results obtained, the behaviour of biopsy samples from the rectal mucosa of the rabbit was compared with that of larger sheets of rectal tissue. Experiments were carried out on female Netherland dwarf rabbits (less than 1 kg in weight) obtained from Nottingham University and allowed free access to food and water. The animal was killed by a blow to the head, the rectum (5 cm of bowel adjacent to the anal

margin) was removed, and after a biopsy specimen had been taken, the muscle layers were removed and a sheet (exposed area 1.925 cm^2) was mounted in a conventional Ussing chamber. The biopsy specimen was set up in the modified Ussing chamber as described above. Both tissues were incubated at 37°C in Krebs bicarbonate saline containing 10 mmol/l glucose and gassed with 95% $\text{O}_2/5\%$ CO_2 . The potential difference was monitored and current applied across the tissue as described above, but in the case of the larger sheet the current pulse was increased to 100 μA . The tissues were allowed to stabilise for 20 minutes after which time readings were taken at one minute intervals.

CHEMICALS

Glucose was obtained from BDH Chemicals Ltd, Poole, England; acetylcholine chloride and amiloride were obtained from Sigma Chemical Co, St Louis, MO 63178, USA.

STATISTICAL ANALYSIS

Results are expressed as mean (SEM) values of the number of observations indicated. The significance of the difference between control and cystic fibrosis tissues was assessed by unpaired Student's t test. In the experiments with the rabbit rectal mucosa, the significance of the difference between the biopsy sample and the larger sheet was determined using an unpaired t test.

Results

HUMAN RECTAL BIOPSY SPECIMENS

Under basal conditions, the biopsy samples generated a potential difference and short circuit current, with the serosal side of the tissue being positive in respect of the mucosal side. The potential difference and short circuit current were significantly lower in the cystic fibrosis tissues ($p < 0.05$ in both cases), although the resistance values were similar in the two groups ($p > 0.05$, Fig 1). The resistance values obtained were smaller than those quoted for larger sheets of human rectal mucosa^{9,10} and this may result from a greater proportion of edge damage in the small sheets of tissue available from biopsy samples. The influence that this might have on the results obtained was examined in rectal tissue from the rabbit (see later). Despite their low resistance values, control tissues responded to the application of acetylcholine (10^{-3} mol/l in the serosal solution) with a rise in short circuit current (mean (SEM) change = 83.0 (16.4) ($n = 12$) $\mu\text{A}/\text{cm}^2$), consistent with a stimulation of chloride secretion (Figs 2 and 3). In contrast, the cystic fibrosis biopsy samples failed to exhibit such a response (mean (SEM) change in short circuit current = -1.4 (3.2) ($n = 5$) $\mu\text{A}/\text{cm}^2$, $p < 0.01$ compared with control tissues).

In both groups of tissues the short circuit current was reduced by mucosal amiloride (10^{-4} mol/l, Figs 2 and 3), which inhibits sodium absorption,¹¹ and the magnitude of this effect was similar in the two groups (control = -37.7

Figure 2: Examples of transmural electrical activity of rectal biopsy specimens from a cystic fibrosis patient (solid symbols) and a control patient (open symbols). Potential difference (Pd) (circles), short circuit current (SCC) (squares), and resistance (R) (triangles) are plotted against time. Acetylcholine (ACh, 10^{-3} mol/l) was added to the serosal solution and amiloride (Am, 10^{-4} mol/l) was added to the mucosal solution at the times indicated by the arrows.

Figure 3: Comparison of the responses of control (○) and cystic fibrosis (●) rectal biopsy specimens to serosal acetylcholine (ACh, 10^{-3} mol/l), mucosal amiloride (Am, 10^{-4} mol/l), and acetylcholine in the presence of amiloride (ACh + Am). The short circuit current (SCC) for each preparation is shown before the addition of the test agent and at the peak of the response.

(7.7) ($n=12$) $\mu\text{A}/\text{cm}^2$; cystic fibrosis = -44.0 (9.3) ($n=5$) $\mu\text{A}/\text{cm}^2$, $p>0.05$). In the cystic fibrosis biopsy samples the decrease in short circuit current induced by amiloride reduced this value to one that was not significantly different from zero ($p>0.05$), but in the control biopsy samples there was a significant residual current (70.7 (6.8) ($n=12$) $\mu\text{A}/\text{cm}^2$, $p<0.001$). The presence of amiloride did not affect the rise in short circuit current induced by acetylcholine in control biopsy tissues (control = 78.9 (18.0) ($n=11$) $\mu\text{A}/\text{cm}^2$, +amiloride = 58.6 (13.6) ($n=11$) $\mu\text{A}/\text{cm}^2$, $p>0.05$; Figs 2 and 3).

RABBIT RECTAL MUCOSA

A comparison of the electrical characteristics of biopsy samples and larger sheets of rabbit rectal mucosa is shown in the Table. Both preparations generated a serosa positive potential difference and short circuit current, with no significant difference between the values obtained. The resistance of the larger sheets was similar to that quoted for rat rectal mucosa.¹² In the biopsy sample, however, tissue resistance was significantly lower, indicating a greater proportion of edge damage in the smaller preparation. Despite their low resistance values, the biopsy samples

Comparison of the electrical activity of a mucosal biopsy specimen and a stripped sheet of rabbit rectum. Basal electrical activity and the short circuit current (SCC) responses to serosal acetylcholine (ACh, 10^{-3} mol/l) and mucosal amiloride (Amil, 10^{-4} mol/l) are given as mean (SEM) of seven observations. An unpaired *t* test was used to assess the significance of the difference between the two preparations

	Biopsy	Sheet	<i>p</i>
Basal PD (mV)	3.0 (0.8)	5.6 (1.2)	>0.05
Basal SCC ($\mu\text{A}/\text{cm}^2$)	82.4 (14.2)	56.7 (14.0)	>0.05
Basal R ($\text{ohm}\cdot\text{cm}^2$)	34.2 (6.6)	111.4 (24.1)	<0.01
ACh ($\mu\text{A}/\text{cm}^2$)	57.8 (15.4)	127.7 (21.9)	<0.05
Amil ($\mu\text{A}/\text{cm}^2$)	-59.9 (17.6)	-53.5 (12.3)	>0.05

PD=potential difference; R=tissue resistance.

were able to generate significant short circuit current responses to acetylcholine, although these were smaller than those obtained in the larger tissue sheets (Table). The effects of amiloride were similar in the two preparations (Table).

Discussion

This study indicates that it is possible to detect an electrical response to acetylcholine in rectal biopsy specimens obtained from a control group of children (Figs 2 and 3). The increased short circuit current is consistent with a stimulation of chloride secretion, which has been shown to be the ionic basis of the cholinergic response in human small intestine¹³ and colon.⁹ Amiloride inhibits electrogenic sodium absorption in the rectum¹¹ leading to a fall in the short circuit current, and this response was observed in all the biopsy samples (Figs 2 and 3). Moreover, amiloride failed to prevent the response to cholinergic stimulation indicating that a change in sodium absorption is not a contributory factor.

Biopsy specimens obtained from children with cystic fibrosis had lower basal potential difference and short circuit current values than those from the control group (Fig 1). The basal short circuit current of cystic fibrosis tissues was abolished by amiloride, indicating that it was due primarily to electrogenic sodium absorption. In control tissues, however, there was a significant residual short circuit current (70.7 $\mu\text{A}/\text{cm}^2$) in the presence of amiloride, similar in magnitude to the difference between the basal short circuit current in control and cystic fibrosis biopsy samples (75.7 $\mu\text{A}/\text{cm}^2$). Such an amiloride-insensitive short circuit current has also been reported in larger sheets of normal human sigmoid colon and rectum^{14,15} and could result from the secretion of chloride ions under basal conditions, since in rat rectal mucosa in vitro the basal short circuit current is reduced by furosemide.¹⁶ Thus, the lower basal short circuit current in cystic fibrosis biopsy samples may result from the absence of this basal chloride secretion, as has been suggested for biopsy samples from the jejunal mucosa.³ In vivo, amiloride abolishes the basal potential difference in both control and cystic fibrosis subjects,⁸ suggesting that the amiloride-insensitive short circuit current in control biopsy specimens is a characteristic of the in vitro preparation. This probably represents chloride secretion resulting from the actions of a variety of secretory stimulants released as a consequence of the mechanical trauma involved in mounting the tissue.¹²

In contrast to the control biopsy specimens, the cystic fibrosis tissues failed to respond to cholinergic stimulation (Figs 2 and 3), indicating that the failure of chloride secretion observed in the small intestine in this disease is also expressed in the rectal mucosa. The fact that amiloride induced similar reductions in short circuit current in control and cystic fibrosis biopsy specimens shows that the cystic fibrosis tissues were viable so that their failure to respond to acetylcholine must represent a specific defect in the chloride secretory mechanism in the

rectum in this disease. While the secretory effects of stimulant laxatives on the rectal mucosa deserve consideration, no single agent was unique to either group. Most cystic fibrosis patients received sodium picosulphate before biopsy but failed to show potentiation after secretagogue stimulation. In vivo studies have shown that the rectal mucosa of cystic fibrosis patients fails to respond to cyclic adenosine monophosphate-mediated secretagogues with a rise in transmural potential difference,⁸ supporting the contention that there is a defect in chloride secretion in the disease. However, the same group could not show a change in potential difference consistent with a chloride secretory response to cholinergic stimulation in either control or cystic fibrosis subjects.¹⁷ A number of factors could contribute to the discrepancy between these results and those reported here. The cholinergic agonist used in vivo was bethanechol, which was applied mucosally, while the biopsy specimens were exposed to acetylcholine on their serosal surface. In addition, the in vivo studies were carried out in adult subjects while the biopsy specimens were obtained from children. It is likely that adult cystic fibrosis clinics would contain a much higher proportion of patients with mild disease and residual pancreatic exocrine function. Haplotype data suggests that pancreatic sufficient and insufficient patients have different mutant alleles and it has been proposed that residual pancreatic enzyme activity in pancreatic sufficient cystic fibrosis patients directly reflects the activity of the mutant gene product.¹⁸ Thus, quantitative differences in ion transport may be expected with the cystic fibrosis pancreatic sufficient allele, which would constitute a dominant phenotype over the more severe pancreatic insufficient mutations that show little or no function. It is also possible that the transport function of the rectum varies with age. A recent study has shown that this is certainly true in the case of sodium absorption, which is reduced with age.¹⁹ These factors do not, however, operate in the jejunum, where it has been shown that mucosal administration of the cholinergic agonist pilocarpine in vivo increases the transintestinal potential difference in normal adults but not in cystic fibrosis adults.⁵

Chloride secretion induced by cholinergic stimulation of the normal intestine is thought to be mediated by a rise in the concentration of cytoplasmic free calcium.²⁰ Cystic fibrosis enterocytes, both in the small intestine³⁻⁶ and the rectum (present study), fail to exhibit a secretory response to cholinergic agonists, and in this respect the intestinal epithelium differs from the airway mucosa where cyclic adenosine monophosphate-mediated secretion is defective¹ but calcium-mediated secretion is normal.²¹ In addition, cyclic GMP mediated secretion also fails in cystic fibrosis small intestine.²² This suggests a fundamental difference in the way in which the chloride secretory process is activated in the two tissues. The cystic fibrosis gene product, the cystic fibrosis transmembrane conductance regulator, is a protein whose function is as yet unknown but which must play a vital role in this process.²³ In both the gut²⁴ and the airway¹

the generation of intracellular messengers seems to be normal in cystic fibrosis and so the cystic fibrosis transmembrane conductance regulator is thought to be concerned with transmitting this signal to the chloride channel. The fact that all three known intracellular signalling systems fail to activate chloride secretion in the cystic fibrosis gut suggests that the transmembrane conductance regulator plays a more central role in this tissue than in the airway, acting either directly on the chloride channel itself or as a common regulatory factor that is the focus of action of all the intracellular mediators.

The studies with rabbit rectal mucosa suggest that a higher degree of edge damage is responsible for the low resistance observed in the biopsy preparation. Nevertheless, this preparation exhibits qualitatively the same pattern of results as that obtained with a larger sheet of rectal mucosa (Table). It may therefore be valid, in the absence of an alternative source of tissue, to use rectal biopsy specimens to determine transintestinal electrical activity.

The electrical response of rectal biopsy specimens to cholinergic stimulation clearly distinguishes between control and cystic fibrosis tissues. The relative accessibility of the rectal mucosa suggests that this test could be of diagnostic value in cases where sweat electrolyte data are ambiguous. It also represents a tissue that could be used to investigate the basic defect in cystic fibrosis.

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