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Acetylcholine induces cytosolic Ca^{2+} mobilization in isolated distal colonic crypts from normal and cystic fibrosis mice

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Abstract

In intestinal biopsies from cystic fibrosis (CF) patients acetylcholine fails to elicit a chloride secretion response, and this observation can be explained by a defect in the Ca^{2+} signalling pathway in CF secretory cells. We tested the hypothesis that in CF intestine, the generation of an intracellular Ca^{2+} signal upon cholinergic stimulation is absent. A transgenic CF mouse model was used. Electrical measurements on intact jejunum and unstripped colon were performed in Ussing chambers. Intact distal colonic crypts were isolated, and the intracellular Ca^{2+} concentration was monitored using the Ca^{2+}-sensitive dye fura-2. Acetylcholine increased the short-circuit current generated by wild-type jejunum and colon, but failed to induce a response in CF tissues. Acetylcholine caused a transient elevation in the intracellular Ca^{2+} concentration in colonic crypts from both wild-type and CF mice; the amplitude and timing of the response in CF crypts was indistinguishable from that in wild-type crypts. The response to acetylcholine was also observed in the absence of extracellular calcium, indicating intracellular stores as the source from which the cytosolic Ca^{2+} concentration increased. We conclude that the absence of a cholinergically-induced secretory response in CF intestine is not due to a defect in the generation of a Ca^{2+} signal in intestinal cells upon cholinergic stimulation.

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Introduction

Cystic fibrosis (CF) is the most common genetic disorder in the Caucasian population. The primary defect of the disease is a mutation in the cystic fibrosis transmembrane conductance regulator (*Cftr*) gene which codes for a cAMP-activated Cl^- channel in epithelial cells (Riordan et al 1989). The most common mutant *Cftr* allele, ΔF508, codes for a misfolded CFTR protein which is not sorted to the proper membrane location (Cheng et al 1990). Consequently, CF cells do not express a cAMP-activated Cl^- permeability, and this leads to clinical symptoms in the digestive tract, for example, meconium ileus, intestinal obstruction syndromes, and pancreatic insufficiency, and in the airways, where defective mucociliary clearance leads to recurrent bacterial infections.

Intestinal Cl^- secretion can be stimulated by cholinergic agonists (Biagi et al 1990; Binder et al 1997) which release Ca^{2+} from intracellular stores (Satoh et al 1995; Williams et al 1997). In small intestinal, colonic and rectal tissues from healthy subjects, the cholinergic agonists acetylcholine and bethanechol elicit a secretory response, but this does not occur in intestinal tissues from CF patients

(Berschneider et al 1988; Taylor et al 1988; Goldstein et al 1991; Hardcastle et al 1991). This is in contrast with the CF airway, where cAMP-mediated Cl^- secretion through CFTR is impaired, but an alternative, Ca^{2+}-activated Cl^- secretion is still present (Boucher et al 1989; Mason et al 1991). In animal models, the severity of the CF phenotype and organ disease (e.g. in small intestine and colon) was found to correlate with the activity of this Ca^{2+}-activated Cl^- conductance (Clarke et al 1994; Rozmahel et al 1996; Wilschanski et al 1996). More interestingly, in rectal biopsies of CF patients the magnitude of a residual Cl^- secretion appeared to vary with a Ca^{2+}-activated Cl^- conductance (Veeze et al 1991, 1994). Restoration of this alternative Cl^- conductance in the human CF intestine could normalize mucosal secretions and therefore alleviate intestinal obstruction syndromes and improve the absorption of nutrients which, in the diseased state, is impaired by thick, viscous mucus (Sinaasappel 1992; Veeze 1992; Robberecht & Sinaasappel 1999). Indeed, it has been suggested that the Ca^{2+}-activated Cl^- channel family, the putative molecular basis underlying the Ca^{2+}-sensitive Cl^- conductance, can be a pharmacological target for therapeutic intervention in CF (Fuller & Benos 2000). The relative lack of experimental data, however, warrants further research.

Based on our observation that the calcium ionophore A23187 evokes small and variable secretory responses in CF jejunal biopsies (Taylor et al 1988; Hardcastle et al 1993), we have put forward the hypothesis that the failure of cholinergically-mediated Cl^- secretion in CF intestine is caused by the absence of an intracellular Ca^{2+} signal upon cholinergic stimulation (Hardcastle et al 1993). We have now tested this hypothesis by measuring the cytosolic Ca^{2+} concentration in intact distal colonic crypts in response to cholinergic stimulation, using the transgenic *Cftr*^{tm2Cam} mouse model possessing the ΔF508 mutation. In this study we combined electrophysiological measurements in intact colonic tissue with measurements of cytosolic Ca^{2+} concentrations in intact colonic crypts.

Materials and Methods

Materials

Cell-Tak cell and tissue adhesive was purchased from Becton Dickinson Labware (Bedford, UK). The acetoxymethyl (AM) ester form of the calcium-sensitive fluorescent dye fura-2 was from Molecular Probes Europe BV (Leiden, The Netherlands). The chloride salt of acetylcholine was from Sigma Chemical Co. (St Louis, MO). Nucleotides, Red-Hot Taq polymerase and other polymerase chain reaction (PCR) reagents were from Advanced Biotechnologies (Epsom, UK). All other chemicals were analytical grade and obtained from commercial suppliers.

Animals and analysis of wild-type and mutant alleles

We used the transgenic CF mouse strain *Cftr*^{tm2Cam} (Colledge et al 1995) in which the ΔF508 mutation was introduced into the *Cftr* gene using a targeting construct containing the 3-bp deletion between nucleotides 1522 and 1524 of *Cftr*. Mice were bred in the Sheffield University Field Laboratories. All mice had free access to standard laboratory animal feed and water, and were killed by cervical dislocation. Adult mice were used, and homozygous ΔF508/ΔF508 mice were compared with wild-type littermates. Mice were genotyped for the ΔF508 mutation by a PCR amplification method (Rommens et al 1990). The PCR primers (CF216F and CF313R) were redesigned to amplify the region surrounding the ΔF508 deletion across the published GENBANK sequences (mouse, M84614; rabbit, U40227; sheep, U20418; human, M28668). The genomic PCR primer sequences were: CF216F: 5′-att aag cac agt gga aga-3′, and CF313R: 5′-ctc atc ata gga aac acc-3′. A crude preparation of genomic DNA was prepared from blood spots collected on Whatman 3MM filter paper and was used as the PCR template. The PCR products were resolved by electrophoresis on a 7% non-denaturing polyacrylamide gel. Gels were visualized by staining with ethidium bromide, and the genotype was obtained from the size (wild-type: 99 bp; mutant: 96 bp) and number of the PCR products.

Electrophysiology

The genotype of the mice was phenotypically confirmed by measuring the acetylcholine-induced secretory response as a rise in short-circuit current (SCC) in intact sheets of proximal jejunum and distal colon mounted in Ussing chambers (aperture = 3 mm^2). Colonic sheets were derived from the region immediately adjacent to the distal colonic segment from which intact crypts were isolated. Potential difference, SCC and tissue resistance were measured as described elsewhere (Hardcastle et al 1999). Acetylcholine was added to the serosal half-chamber to give a final concentration of 1 mM.

Isolation of colonic crypts

Intact colonic crypts were isolated from distal colon by incubating an everted 2–3 cm segment, taken from the region immediately adjacent to the rectum, in 15 mM ethylenediamine-tetraacetic acid (EDTA) followed by mechanical vibration as described in detail elsewhere (Hardcastle et al 1999). Isolated crypts were washed thoroughly and resuspended in 10 mL oxygenated HEPES-buffered Krebs saline (143 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM D-glucose, and 20 mM HEPES/Tris at pH 7.4).

Microfluorimetry

Isolated crypts were transferred to a Cell-Tak pretreated coverglass and allowed to settle for 30 min at room temperature in an oxygenated atmosphere. The coverglass was mounted in a PDMI-2 temperature-controlled incubation chamber (Medical Systems Corp., Greenvale, NY) on the stage of an inverted microscope. A RatioMaster photometric system from Photon Technology International (Surbiton, UK) was used. We used the calcium-sensitive fluorescent dye fura-2. Details of obtaining 340 and 380 nm excitation wavelengths and signal detection have been previously described (Hardcastle et al 1999). Crypts were superfused with oxygenated Krebs saline at 37°C. In Ca^{2+}-free Krebs, $CaCl_2$ was omitted and 0.1 mM ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added. Perfusion with experimental solutions started after a stable baseline was obtained. The fura-2 signals are expressed in dimensionless 340 nm/380 nm excitation wavelength (340/380) ratios.

Statistics

Data are expressed as mean ± s.e.m. unless indicated otherwise, with the number of preparations in parentheses. Statistical significance was assessed using the Student's *t*-test for unpaired data (two-tailed) and one-way analysis of variance, where appropriate. $P < 0.05$ was considered significant.

Results

Table 1 shows that basal SCC values in wild-type and CF jejunum did not differ significantly ($P = 0.89$). In distal colon, however, basal SCC values for the CF genotype were eightfold lower than for the wild-type genotype ($P = 0.004$), reflecting the reduction in basal net electrogenic ion transport in CF colon. Serosally-added acetylcholine (1 mM) increased the SCC generated by wild-type jejunum and distal colon by 38 ± 11 (n = 6) and 16 ± 4 (n = 10) μA cm^{-2}, respectively. However, acetylcholine failed to induce a response in CF jejunum and colon (Figure 1), thus confirming the secretory defect in the intestine of the CF mouse model.

Background 340/380 ratios in wild-type and CF colonic crypts were virtually identical: 1.31 ± 0.046 (n = 25) and 1.30 ± 0.026 (n = 14) (means ± s.d., $P = 0.62$), respectively. This demonstrates the stability of the instrumentation, and validates a direct comparison of 340/380 ratios measured on different preparations.

Table 1 Basal short-circuit currents (μA cm^{-2}) in intact jejunum and colon from wild-type and CF mice.

	Basal short-circuit current (μA cm^{-2})	
	Jejunum	Colon
Wild-type	16.9 ± 2.0 (n = 6)	63.7 ± 9.1 (n = 10)
CF	17.8 ± 5.8 (n = 5)	8.3 ± 6.8 (n = 4)*

*$P < 0.01$ for wild-type vs CF colon.

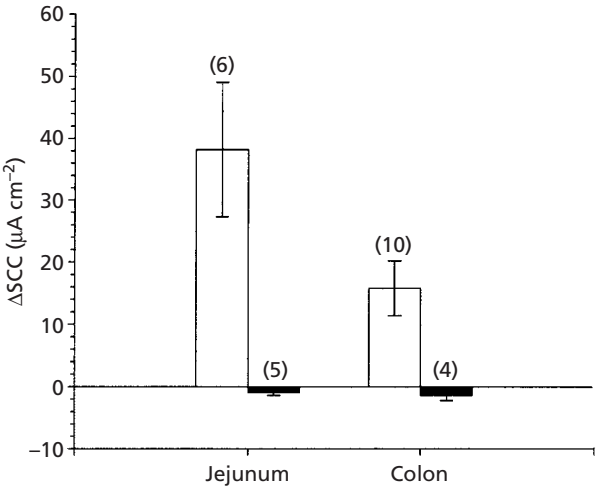

Figure 1 Effect of 1 mM acetylcholine on the short-circuit current (SCC) generated by wild-type and CF mouse jejunum and distal colon. Each bar represents mean ± s.e.m. of the number of observations indicated in parentheses. Student's *t*-test for unpaired data was used to assess the significance of the differences between wild-type (□) and CF (■) tissues (wild-type vs CF jejunum, $P < 0.01$; wild-type vs CF colon, $P < 0.05$).

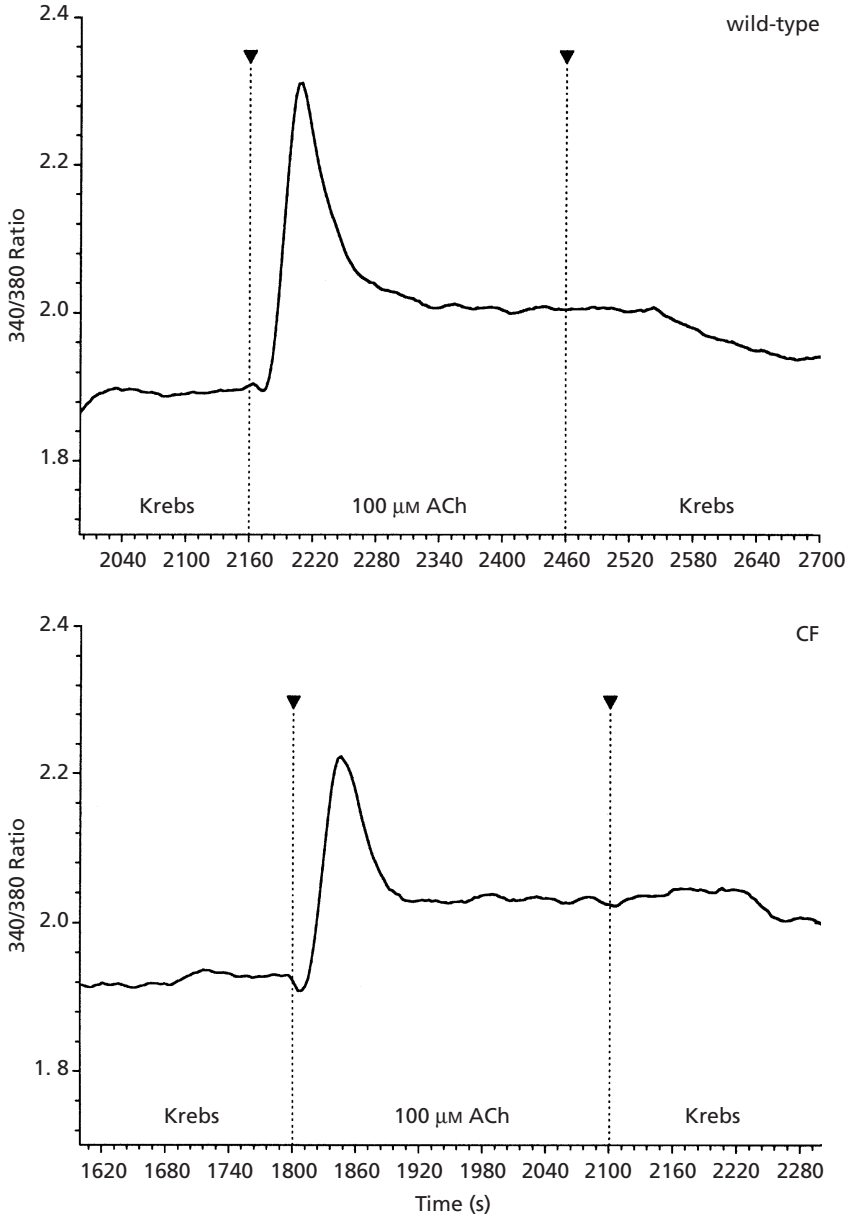

Figure 2 Effect of 100 μM acetylcholine (ACh) on fura-2 fluorescence in a single isolated distal colonic crypt of wild-type and CF mouse distal colon. The extracellular Ca^{2+} concentration was 2.5 mM. The tracings depicted are typical for observations in ten wild-type and eight CF crypts.

Baseline 340/380 ratios, corrected for background fluorescence, were similar in wild-type and CF colonic crypts: 1.6 ± 0.2 (n = 10) vs 1.7 ± 0.2 (n = 8), respectively (Figure 2). This corroborates a previous study using human tissue where we found no differences between cytosolic Ca^{2+} levels in isolated normal and CF enterocytes (Hitchin et al 1991). Acetylcholine (100 μM) induced a transient change in the 340/380 ratio. In wild-type crypts, the ratio increased from baseline to a peak value of 2.0 ± 0.3 (n = 10). An identical peak value was observed in CF crypts: 2.0 ± 0.2 (n = 8). The peak value of the response in wild-type crypts appeared 67 ± 6 s (n = 10) after the start of the acetylcholine superfusion. In CF crypts a similar time interval was observed: 79 ± 7 s (n = 8), which was not significantly different from that observed in wild-type crypts ($P = 0.21$). In a previous study, using colonic crypts from a Swiss MF1 mouse strain, we measured a time-to-peak value of 90 ± 12 s

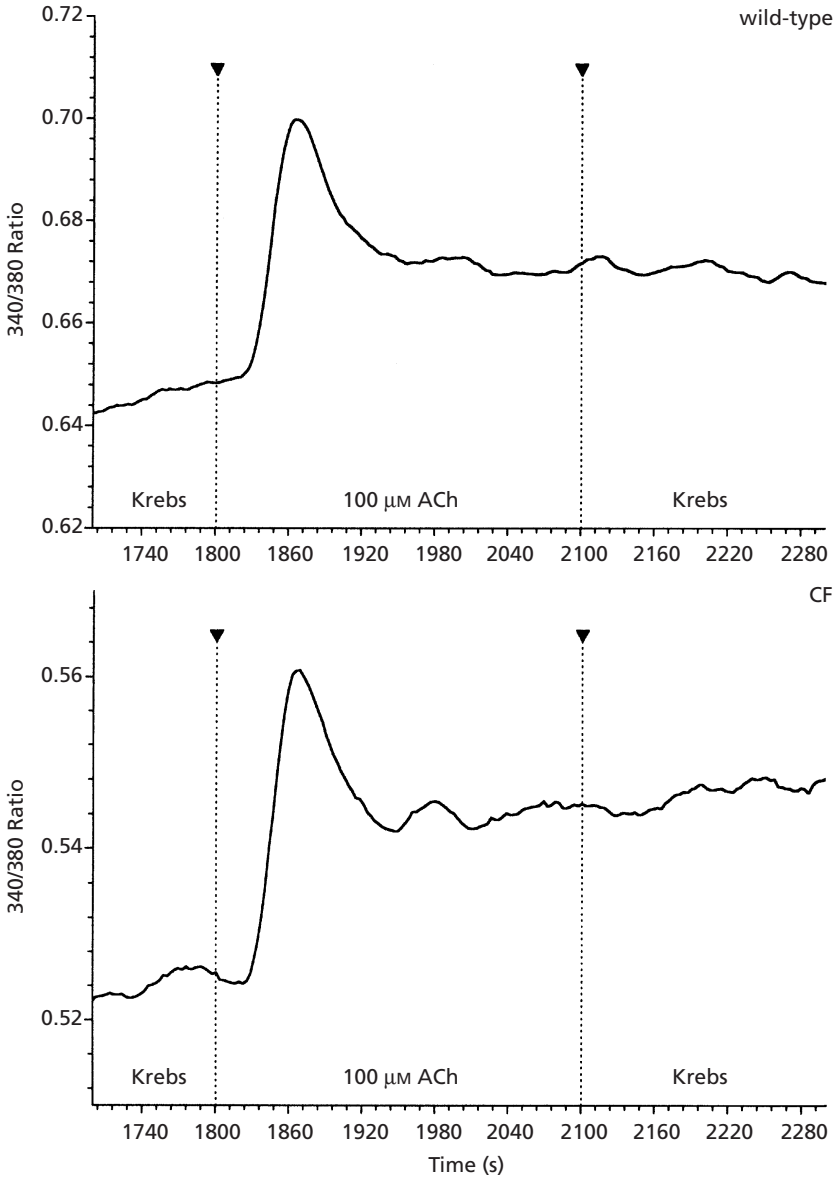

Figure 3 Effect of 100 μM acetylcholine (ACh) on fura-2 fluorescence in a single isolated crypt of wild-type and CF mouse distal colon in the absence of extracellular Ca^{2+}. $CaCl_2$ was omitted, and 0.1 mM EGTA was added to the Krebs superfusion buffer. The tracings depicted are typical for observations in four wild-type and six CF crypts.

(n = 5) (Hardcastle et al 1999), and this value did not differ significantly from the values measured under identical conditions in crypts from our transgenic mouse strain ($P = 0.15$, one-way analysis of variance). After the peak response and still in the presence of acetylcholine, the 340/380 ratio fell to a value of 1.8 ± 0.2 (n = 9) in wild-type and 1.8 ± 0.2 (n = 7) in CF crypts. The response to acetylcholine was also observed in the absence of extracellular calcium in wild-type crypts (n = 4) as well as CF crypts (n = 6) (Figure 3), indicating intracellular stores as the source from which the cytosolic Ca^{2+} concentration increased.

Discussion

Acetylcholine failed to elicit a secretory response in the jejunum and colon of our transgenic ΔF508 CFTR mouse model, a result that corroborates our observations on intestinal biopsies from CF patients (Taylor

et al 1988; Hardcastle et al 1991). Acetylcholine-induced cytosolic Ca^{2+} mobilization in colonic crypts, however, functions normally in CF mouse intestine, and we conclude that the failure of cholinergic agonists to induce a secretory response in CF intestine is not due to the absence of a cytosolic Ca^{2+} signal in intestinal cells in response to cholinergic stimulation. Similar results have been found in a CF pancreatic cancer cell line (Galietta et al 1994; Roch et al 1995) and in human CF airway epithelia (Mason et al 1991; Rugolo et al 1993) using nucleotide agonists. However, in CF airway epithelia the intracellular Ca^{2+} response caused an activation of transmembrane K^+ and Cl^- transport (Mason et al 1991; Rugolo et al 1993), and this effect was virtually absent in our preparations from CF mouse intestine.

In healthy intact intestinal preparations, increases in the SCC generated upon stimulation with Ca^{2+}-mediated secretagogues have been reported (Berschneider et al 1988; Taylor et al 1988; O'Loughlin et al 1991; Clarke et al 1994; Grubb 1997), and this effect was absent in CF human intestinal biopsies (Berschneider et al 1988; Taylor et al 1988; O'Loughlin et al 1991) and intact intestine of *Cftr*^{tm1UNC} and S489X *Cftr* knockout mice (Clarke et al 1994; Grubb 1997). The absence of a Ca^{2+}-mediated secretory response in CF intestine suggests that CFTR is involved in Ca^{2+}-activated Cl^- secretion. This is corroborated by the observation that the Ca^{2+} ionophore-induced secretory response in intact intestine of control mice is insensitive to the Cl^- channel blocker 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (Anderson & Welsh 1991). Several groups have proposed a model where the cholinergic secretory effects are not mediated through an apical Ca^{2+}-activated Cl^- channel, but rather through the activation of a basolateral Ca^{2+}-activated K^+ channel, with subsequent cell hyperpolarization and increase in the driving force for Cl^- exit through non-stimulated Cl^- channels (Anderson & Welsh 1991; Valverde et al 1994; Greger et al 1997; Grubb 1997; Inoue et al 1997), for example, CFTR. Although this model explains the absence of a Ca^{2+}-activated Cl^- current in CF tissues, it does not account for the observations of residual Ca^{2+}-activated Cl^- currents in intestinal biopsies from a subclass of CF patients (Taylor et al 1988; Veeze et al 1991), and for the UTP-(mediated via intracellular Ca^{2+}) and Ca^{2+} ionophore-induced Cl^- conductances measured in-vivo in rectum and in-vitro in isolated crypt cells of specific long-living strains of *Cftr* knockout mice (Rozmahel et al 1996; Wilschanski et al 1996) which do not express a functional CFTR. These results indicate that, at least in some circumstances, Ca^{2+}-activated intestinal Cl^- secretion is not mediated through CFTR. Genetic modifiers, as yet unidentified, are thought to be involved in this alternative Cl^- conductance (Rozmahel et al 1996; Wilschanski et al 1996).

This study demonstrates that in normal and CF colon cholinergic stimulation generates an increase in cytosolic Ca^{2+} by releasing Ca^{2+} from intracellular stores, indicating that in CF colon the defect in cholinergic Cl^- secretion is located downstream from the event of cytosolic Ca^{2+} mobilization. A normal Ca^{2+} signal is observed in CF tissues but is not translated into a secretory event, suggesting that CFTR is an absolute requirement for the secretion of Cl^- by the colon, making Ca^{2+}-activated Cl^- channels a less interesting candidate for therapeutic intervention in CF.

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