The genetic advantage hypothesis in cystic fibrosis heterozygotes: a murine study

A. W. Cuthbert, J. Halstead, R. Ratcliff *, W. H. Colledge* and M. J. Evans*

*Department of Pharmacology and *Wellcome/CRC Institute of Cancer and Developmental Biology, Department of Genetics, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK*

1. The ΔF508 mutation of the cystic fibrosis (CF) gene is of high frequency in man (1 in 25) and in homozygotes causes cystic fibrosis. It is suggested that cystic fibrosis heterozygotes withstand secretory diarrhoea better than normal individuals and so are genetically advantaged. This hypothesis has been examined by measuring electrogenic chloride secretion in gut epithelia of normal and heterozygous CF mice.

2. Chloride secretory responses of normal and heterozygous colonic epithelia to forskolin, vasoactive intestinal polypeptide (VIP), isoprenaline, cholera toxin, heat-stable enterotoxin (STa), guanylin, carbachol and lysylbradykinin were examined. No significant differences in responses of tissues of the two genotypes were found.

3. Responses of normal and heterozygous ileal epithelia to forskolin and glucose were investigated. Heterozygous tissues responded as well as normal tissues.

4. Frusemide (furosemide) caused virtually identical inhibition of the chloride secretory responses to forskolin in colonic epithelia of both genotypes.

5. No evidence to support the genetic advantage hypothesis in ileal or colonic epithelia of the null CF mouse has been found, at least for acute responses. If the hypothesis is true then either (a) other non-cystic fibrosis transmembrane conductance regulator (non-CFTR) transport processes are involved, (b) prolonged exposure to secretagogues is required, or (c) ΔF508 CFTR is responsible for the protective effect.

The high frequency of the cystic fibrosis (CF) gene within the gene pool, *circa* 1 in 25 amongst Caucasians (Collins, 1992), might be explained if heterozygotes enjoy, or have enjoyed in the past, some genetic advantage. The most frequent hypothesis given is that heterozygotes are protected against the ravages of secretory diarrhoea, and less likely to succumb to the debilitating consequences (Hansson, 1988; Baxter, Goldhill, Hardcastle, Hardcastle & Taylor, 1988; Field & Semrad, 1993; Guggino, 1994; Quinton, 1994; Chao, de Sauvage, Dong, Wagner, Goeddel & Gardner, 1994). The hypothesis is logical since the CF gene codes for an epithelial chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR), essential for intestinal fluid secretion. Furthermore, CF intestinal epithelia, either from human biopsy or the CF mouse model, fail to show chloride secretion in response to a variety of secretagogues (Berschneider *et al.* 1988; Taylor, Baxter, Hardcastle & Hardcastle, 1988; O'Loughlin *et al.* 1991; Cuthbert *et al.* 1994*b*; Cuthbert, MacVinish, Hickman, Ratcliff, Colledge & Evans, 1994*c*; Cuthbert, Evans, Colledge, MacVinish & Ratcliff, 1994*a*). While CF heterozygotes are not threatened by lethal disease they possess only a single allele capable of generating CFTR, which may limit the maximal secretory capacity. Thus in epidemics, such as cholera or caused by enterotoxin-generating *Escherichia coli*, heterozygotes may be expected to have a better chance of survival, leaving the population enriched in carriers. Mice in which the CF gene has been eliminated (Ratcliff *et al.* 1993) provide a unique opportunity for testing the genetic advantage hypothesis. Since such individuals carry null mutations there is no doubt that only one allele is capable of generating protein, unlike human heterozygotes in which one allele produces an altered protein with or without only minor functional capability.

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METHODS

CF mice

The generation of heterozygous mice carrying the mutant *cftr* gene has been described previously (Ratcliff *et al.* 1993). Following genotyping, normal, heterozygous and CF animals were segregated. In this study fifty-two animals (24·1 ± 5·9 g) with the normal genotype (+/+) and fifty heterozygotes (26·9 ± 8·0 g; +/−) were used.

Measurement of electrogenic chloride secretion

Mice were killed by exposure to 100% CO_2 and the small and large intestine removed immediately. One, and sometimes two, pieces of distal colon or mid-ileum, 7 mm long, were removed and were immediately opened and cleaned. The longitudinal and circular smooth muscle layers were removed by dissection and the epithelium mounted in an Ussing chamber, sandwiched between layers of parafilm to cushion the tissue. The window area was 20 mm^2. Short circuit current (SCC) was measured by a standard procedure using a dual voltage clamp (World Precision Instruments), with current compensation for series resistance between the voltage-sensing electrodes. A total of seventy-six normal epithelia and eighty epithelia from heterozygous animals were used in these studies. The epithelia were bathed on both sides with 20 ml Krebs–Henseleit solution (KHS) gassed with 95% O_2–5% CO_2 and maintained at 37 °C. Throughout values are stated as the means ± standard error of the mean. To compare values Student's t test was used and $P < 0{\cdot}05$ was considered significant.

Chemicals and solutions

Cholera toxin and heat-stable enterotoxin (STa) were obtained from Sigma Chemical Co., and guanylin was custom-synthesized for another study (Cuthbert *et al.* 1994*b*); all the other substances and salts were from the usual sources and were of the highest grades. The composition of the KHS was as follows (mM): NaCl, 118; KCl, 4·7; $CaCl_2$, 2·5; $MgSO_4$, 1·2; KH_2PO_4, 1·2; $NaHCO_3$, 25·0; and glucose, 11·1.

RESULTS

Stimulation of chloride secretion by agents increasing cyclic nucleotides in colonic epithelia

Epithelial CFTR chloride channels are activated by agents which increase intracellular cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) concentrations. Forskolin, acting directly on adenylate cyclase, and vasoactive intestinal polypeptide (VIP) acting directly through its receptors were used to stimulate chloride secretion via cAMP in normal and heterozygous colonic epithelia. Both agents produced well-maintained responses so that cumulative addition of both agents was possible. There were no significant differences between the maximal responses or the concentration–response relationships for normal or heterozygous tissues using either of the agonists (Fig. 1). Furthermore, the maximal secretory effect of both agonists was the same, suggesting that the same transport process(es) can be maximally activated by both agonists. Integration of the response curves showed that heterozygotes transported ~2·7 μequiv cm^{-2} in 1 h. Two other agents known to increase cAMP, isoprenaline and cholera toxin, were also investigated. For the former only a low concentration and a supramaximal concentration were used. Peak responses to isoprenaline were not different in tissues of the two genotypes at either concentration (Fig. 3*A*). Cholera toxin needs to be processed by the cell before the modified A-subunit can affect adenylate cyclase, a process taking several hours. In the mouse colon there was a delay of 90 min before the current slowly increased, usually reaching a plateau in 4 h (Fig. 2). The maximal responses to a fixed concentration of toxin (10 μg ml^{-1}) were variable but again there was no

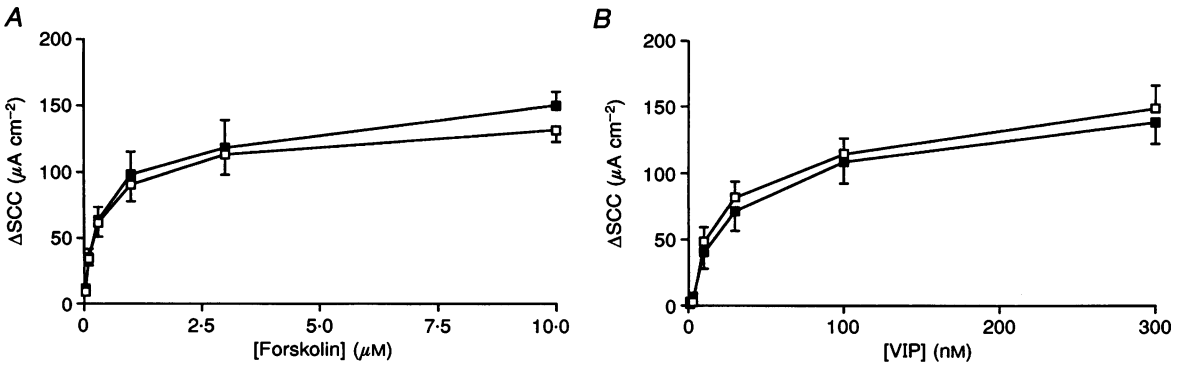

Figure 1. Increases in SCC caused by forskolin (*A*) and VIP (*B*) in colonic epithelia from normal (□) and heterozygous (■) mice

Both mean values and standard errors of the mean are given. In *A* forskolin was added cumulatively in 9 normal and 8 heterozygous tissues, 10 min being allowed for equilibration at each concentration. A further 19 measurements for each type of tissue were made using only 10 μM forskolin, making n values for this concentration only 28 for normal and 27 for heterozygous tissues. In *B* the results are for 5 tissues of each type, VIP being added cumulatively. The differences between the pairs of curves is not significant for the tissues of the two genotypes.

significant difference between the responses of normal and heterozygous colon, with the largest increases comparable to the maximal responses achieved with forskolin. Heterozygous tissues transported ~6 μequiv cm^{-2} in 4 h.

Heat-stable enterotoxin (STa) and guanylin were chosen as agents acting through guanylate cyclase, guanylin being the endogenous ligand for STa receptors. These receptors are located on the apical surfaces of epithelia and the responses are rapid. Lack of availability of the peptides restricted the concentrations that could be used. A single submaximally effective concentration of each peptide was employed and no differences were found in the responses of normal and heterozygous colon (Fig. 3*B*).

Stimulation of chloride secretion in the colon by agents which increase intracellular Ca^{2+}

Agonists such as carbachol (CCh) increase $[Ca^{2+}]_i$, which activates basolateral Ca^{2+}-sensitive K^+ channels, the consequent cellular hyperpolarization increasing the driving force for chloride exit through the apical surface chloride channels. The hypothesis predicts that there will be fewer of these in heterozygous than in normal epithelia. Cumulative response curves to CCh could not be obtained because of rapid desensitization; consequently an alternative method was used. This comprised adding a low concentration of CCh causing little desensitization, washing this away immediately and 30 min later adding a supramaximal concentration. The resultant transient increase in SCC was analysed to give full concentration–response curves. The principle of this method assumes that diffusion of the drug through the stationary layer governs the time course of the biological response. The responses at different concentrations of CCh are obtained by overlaying the transient response to the supramaximal concentration by a diagram relating the values of C_t/C_0 (concentration of drug at the receptors at time t/concentration of drug at the receptors at $t = 0$) against time, arranging the transient at the appropriate value of L^2/D (where L is the equivalent thickness of the stationary layer and D is the diffusion constant of the drug). The method is described in full elsewhere (Cuthbert & Dunant, 1970). A diagram showing the analysis of a transient is given as the inset in Fig. 4; it is to be noted that immediately after the supramaximal concentration of agonist is applied the SCC is momentarily reduced, perhaps as a result of K^+ secretion. In Fig. 4, data for the analysis of all transient responses are given for tissues of both genotypes, but no significant differences were found. The peak responses to the sub- and supramaximal concentration of CCh are given in Fig. 3*D*; also included is the fall in SCC seen at the commencement of each transient. None of these are significantly different in normal and heterozygous tissues.

Lysylbradykinin (LBK) increases chloride secretion in the intestine both by raising intracellular Ca^{2+} and by increasing cAMP; however, the responses rapidly peak and decline to a plateau level. Only one concentration was used in comparing normal with heterozygous tissues, namely 0·1 μM. In four separate experiments in normal colon the responses were no greater at 10 μM compared with 0·1 μM LBK (140·0 ± 23 $\mu A\ cm^{-2}$ at 0·1 μM and 144·0 ± 28 $\mu A\ cm^{-2}$ at 10 μM). The data given in Fig. 3*C* show that heterozygous colon and tissues from wild-type animals respond equally well. Indeed, two responses in

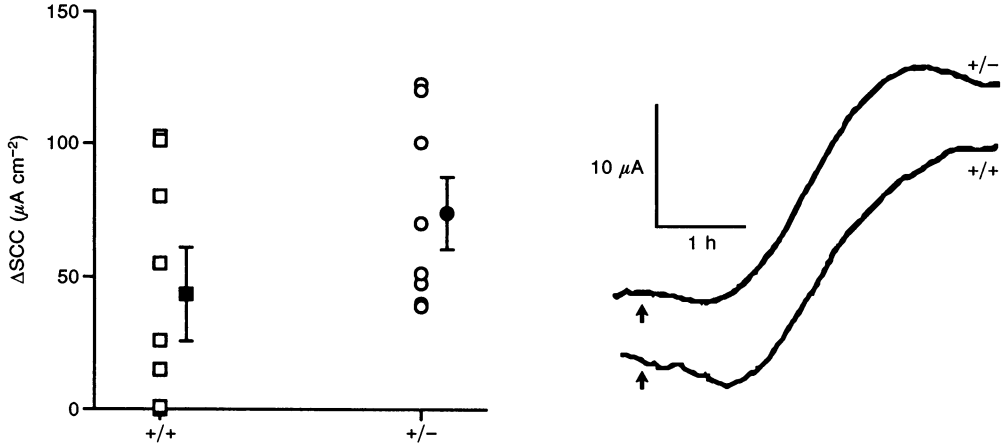

Figure 2. Increases in SCC in response to cholera toxin (10 $\mu g\ ml^{-1}$) applied to the apical surface of colonic epithelia from normal (+/+; □) and heterozygous (+/−; ○) mice

Experiments were performed in pairs with one tissue of each type ($n = 8$ for both). Arrows on the specimen traces (right panel) indicate the time at which the toxin was added. SCC increases were taken as the difference between the lowest value of the SCC and the highest value achieved within 4 h. A scatter diagram (left panel) of the responses together with the mean values (filled symbols) and standard errors of the mean (bars) are given. The mean values were not significantly different.

heterozygous colon were very large (mean 297 μA cm^{-2}) and were outside the normal distribution of all the other responses measured with LBK. The data in Fig. 3*C* are presented both with and without this pair. The peak responses to LBK were similar to those achieved with forskolin and VIP.

Responses in normal and heterozygous ileal epithelia

Identical experiments to those described earlier with forskolin were made with ileal epithelia taken from the mid-region. In this instance the response of heterozygous ileal tissue was larger than that of normal tissue at the highest forskolin concentrations used. In normal tissue the response to 10 μM forskolin was 78·9 ± 6·9 μA cm^{-2} ($n = 10$), whereas in heterozygotes SCC increased by 108·5 ± 8·5 μA cm^{-2} ($n = 10$). These values were significantly different ($P < 0.05$). Heterozygous ileal epithelia transported 2·2 μequiv cm^{-2} during the 1 h required to determine the concentration–response curves.

Responses of ileal epithelium to glucose addition to the apical surface were also investigated. Human CF ileum shows an exaggerated SCC response to apical glucose addition due to stimulation of carrier-mediated electrogenic sodium transport (Hardcastle, Hardcastle & Taylor, 1993). This process is antisecretory and it was important to determine whether it also occurred in heterozygotes. Cumulative addition of glucose in eight steps between 0·5 and 30·0 mM increased SCC by 135·3 ± 35·5 and 134·0 ± 36·4 μA cm^{-2} in normal and heterozygous tissues, respectively ($n = 5$ in both groups), with no difference between the concentration–response curves. No attempt was made to correct this result for tonicity changes caused by adding glucose only apically to initially glucose-free solution

Inhibition of chloride secretion by frusemide in normal and heterozygous colonic epithelia

In CF colon forskolin causes a reduction in SCC due to the stimulation of K^+ secretion (Cuthbert *et al.* 1994*c*), a

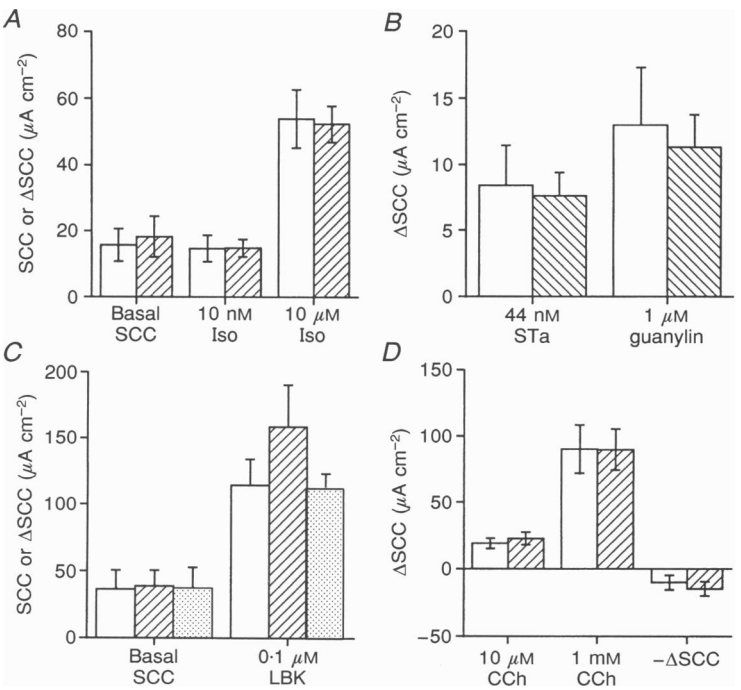

Figure 3. Composition of responses in normal and heterozygous colons

A, basal SCC and peak SCC increases in response to isoprenaline (Iso, 10 nM and 10 μM), added cumulatively. Values for 6 normal (□) and 6 heterozygous (▨) colonic epithelia are given. Isoprenaline was added basolaterally to the epithelia in the presence of 10 μM phentolamine to avoid any alpha effects. *B*, peak SCC increases in response to 44 nM STa (added apically) in 6 normal (□) and 6 heterozygous (▧) tissues, and also SCC increases in response to 1 μM guanylin in 8 normal and 7 heterozygous tissues. *C*, values of basal SCC and the peak SCC increases in response to 0·1 μM lysylbradykinin (LBK, added basolaterally) in 8 normal (□) and 8 heterozygous (▨) colonic epithelia. Two of the heterozygous colonic samples responded particularly well, the SCC increases being more than the mean value plus 4 times the standard error of the remaining 6 values. The values for 6 heterozygous epithelia with the results from the 2 high responders removed are shown (▩). *D*, SCC increases obtained separately in response to 10 μM and 1·0 mM CCh (added basolaterally), and also the transient fall in SCC which occurred when 1·0 mM CCh was added in 5 normal (□) and 7 heterozygous (▨) epithelia. None of the differences between values for normal or heterozygous tissues was significantly different in *A*–*D*.

process normally masked by the increase due to massive chloride secretion. Frusemide blocks both of these transport processes, as the cotransporter is responsible for both K^+ and Cl^- entry into the cell. It is possible that the virtually identical responses of tissues of the two genotypes to forskolin is fortuitous and results from very different K^+ secretory capacities. If so the inhibitory effectiveness of frusemide will be different in tissues of the two types. To measure the effects of frusemide individual colonic samples were exposed to 10 μM forskolin every 90 min for 6 h, washing out after each addition until the baseline value was achieved. In alternate applications tissues were pre-exposed to frusemide, either 15 or 100 μM, 15 min before forskolin was added. Responses to forskolin alone were not significantly different on repeated appplication, and neither were the mean responses in normal and heterozygous tissues. Frusemide (15 μM) caused 41·0 $\pm$ 5·6 and 39·0 $\pm$ 8·4% inhibition of the forskolin response in normal and heterozygous colon, while at 100 μM inhibition was 61·6 $\pm$ 2·4 and 59·5 $\pm$ 5·0%, respectively, all values being for ten separate experiments. Thus, no significant difference in the activity of the inhibitor was found with tissues of the two genotypes.

DISCUSSION

As given in the introduction, the most favoured hypothesis for the genetic advantage in CF heterozygotes is their ability to deal with the debilitating effects of secretory diarrhoea. Only modest evidence is available in support of the hypothesis. For example, reduced sweat secretion in heterozygotes (Behm, Hagiwara, Lewiston, Quinton & Wine, 1987) and the reduced rate of ileum potential increase following pilocarpine application in CF carriers (Hardcastle *et al.* 1993). Chloride secretory responses in the murine CF colon to forskolin, carbachol, guanylin and lysylbradykinin are virtually non-existent (Cuthbert *et al.* 1994*a*, *b*, *c*), a pattern not different from that of the human CF gut epithelia (Baxter *et al.* 1988; Berschneider *et al.* 1988; Taylor *et al.* 1988; O'Loughlin *et al.* 1991) indicating that CFTR is essential for the secretory process. No instance has been found in which murine heterozygous epithelia respond less well than normal wild-type tissue. With forskolin (and VIP) it is clear that the maximal secretory response was achieved and this, together with the virtually identical degree of inhibition with frusemide, indicates that, quantitatively and qualitatively, the chloride secretory activity is identical in tissues of the two genotypes, at least following acute exposure to secretagogues. Evidence produced with other agonists corroborates the findings with forskolin.

It is possible to discriminate between normal, heterozygous and CF murine intestine using *in situ* hybridization for *cftr* mRNA (Trezise, Romano, Ratcliff, Higgins, Evans & Colledge, 1993). Intestinal crypt cells produce high amounts of CFTR compared with airway epithelial cells, yet the cells have only a brief lifetime in the mouse (68 h). Why crypt cells should produce high amounts of CFTR for such a brief period of function is unknown, but it is possible that the reservoir of protein is

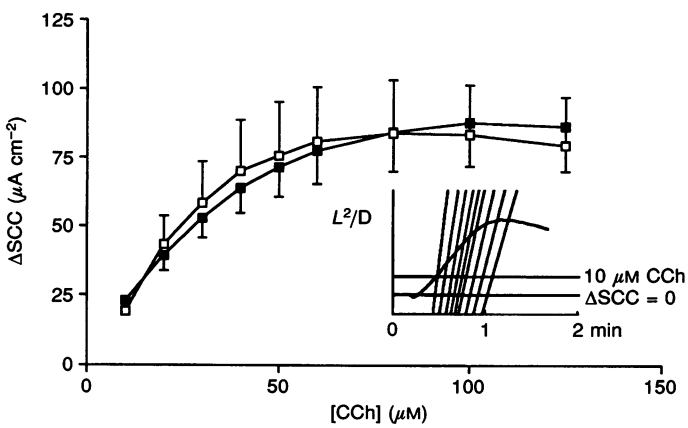

Figure 4. Concentration–response curves to CCh determined from transients for 5 normal (□) and 7 heterozygous (■) epithelia

Mean values plus standard errors of the mean are given. The inset shows the analysis of a transient response to a supramaximal concentration of 1 mM CCh applied to the basolateral side at $t = 0$. The tissue had been previously exposed to 10 μM CCh, which produced a peak response as indicated by the horizontal line. The grid of sloping lines are values of L^2/D (where L is the equivalent thickness of the stationary layer and D is the diffusion coefficient of CCh) against time for values of C_t/C_0 (from left to right) of 0·01, 0·02, 0·03, 0·04, 0·05, 0·06, 0·08, 0·1 and 0·125. Note that neither the value of L or D need be known, but the correct value of L^2/D is found by arranging the value for the response to 10 μM CCh to coincide with the C_t/C_0 value of 0·01 (i.e. 10 μM/1·0 mM). Then, provided the point at which the drug was added is made to coincide with $t = 0$, the responses at other concentration ratios can be read from the transient.

so large that even in heterozygous cells the amount of functional protein in the apical membrane is the same as in normal cells. Nevertheless, in a situation where only one allele can generate a message coding for the apical chloride channel the ability to secrete chloride was unimpaired. The physiological logic of the genetic advantage hypothesis in CF is appealing since a ~2% better survival rate in diarrhoeal disease would maintain the frequency of the CF gene at its present level (Knudson, Wayne & Hallet, 1967). After this paper was submitted a report appeared (Gabriel, Brigman, Koller, Boucher & Stutts, 1994) showing that cholera toxin gave less electrogenic chloride secretion in heterozygous mouse jejunum compared with wild-type tissues, but the difference was not apparent until the heterozygote response faded after 4 h when ~2·3 μequiv cm^{-2} had been transported. This too suggests there is a channel reserve which can be recruited after prolonged stimulation and is smaller in heterozygotes. The question remains whether in ΔF508 heterozygotes, in which the reserve may contain normal and mutant protein, the same result would occur or the reserve be more easily depleted. Finally cAMP not only stimulates chloride secretion but also inhibits electroneutral sodium chloride absorption. It would seem that measurement of actual fluid secretion in ΔF508 hetrozygotes is desirable when these become available.

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