Altered sensitivity to amiloride in cystic fibrosis. Observations using cultured sweat glands

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1 Using cultured epithelia from sweat glands, derived from both cystic fibrosis and normal subjects, the relationship between amiloride concentration and the inhibition of electrogenic sodium transport was measured, under short circuit conditions.

2 The K_d for amiloride in cultures from normal subjects was $0.64 \mu\text{M}$ ($n = 6$) while in cultures derived from CF patients the value was $1.07 \mu\text{M}$ ($n = 4$). The values were significantly different ($P < 0.02$, ANOVA).

3 In cultures from normal sweat glands, bathed in solutions free of permeable anions (chloride/bicarbonate), the K_d for amiloride rose to a value greater than found in CF tissues ($2.3 \mu\text{M}$). In CF epithelia subject to the same conditions the abnormally high value increased further, so that in solutions without permeable anions normal and CF cultures behaved similarly.

4 In cultures derived from normal and CF tissues lowering the sodium concentration to 10 mM also lowered the K_d for amiloride, however the shift was greater for CF cultures.

5 Several possible explanations for the results are discussed. The most probable is that the relatively more positive apical membrane potential in CF epithelia opposes the interaction of amiloride with the sodium channel, implying that complex formation is potential sensitive.

Keywords amiloride sweat glands cystic fibrosis epithelial ion transport

Introduction

Recently, it was shown that cultured epithelial sheets derived from human sweat glands showed ductal properties in respect of their transporting activity (Brayden *et al.*, 1988). Under short circuit conditions virtually the whole current was shown to be due to electrogenic sodium absorption which was sensitive to amiloride. Cultures derived from whole glands or from the secretory coils alone had the same properties, indicating that transformation or de-differentiation occurred in culture. In this study we have investigated the precise quantitative relationship between amiloride concentration and the inhibition of the sodium current. Furthermore we have compared the data obtained with cultures

from normal tissues and those from tissues of patients with cystic fibrosis (CF). The major finding is that the macroscopic affinity for amiloride is significantly reduced in CF.

Methods

Patients

Skin samples from above the sternum were taken from patients undergoing bypass surgery, heart–lung transplantation or from heart–lung donors. Ethical permission was granted by the Huntingdon Health Authority. The CF patients had an age range of between 11 and 33 years.

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Experimental design

Cultures of whole normal and CF sweat glands were produced exactly as described before (Brayden *et al.*, 1988). The cultured monolayers were prepared for short circuit current (SCC) recording according to previous methods (Cuthbert *et al.*, 1987). The epithelial area exposed was 0.2 cm² and each side of the tissue was bathed in Krebs-Henseleit solution (KHS), 20 ml at pH 7.4. The bathing solution was gassed with 95% O₂: 5% CO₂ and maintained at 37°C, unless otherwise stated. Cumulative concentrations of amiloride from 1 nM up to 44.5 µM were added to the apical bathing solution in a stepwise manner at 5 min intervals. At the highest concentrations used no further changes in SCC occurred. Fractional inhibition of the amiloride sensitive SCC at each amiloride concentration was calculated from the experimental data and modelled using the logistic function (De Lean *et al.*, 1978), described by

$$I = \frac{C^n}{K_d^n + C^n}$$

where *I* is the fractional inhibition of SCC, *C* is the amiloride concentration, *K_d* the macroscopic dissociation constant and *n* the Hill slope.

For illustrative purposes separate or pooled datasets were fitted using a non-linear least squares programme. This was performed using the Harwell Library Subroutine VBO1A on the Cambridge IBM 3081. When a number of identical experiments was performed on multiple cultures originating from the same skin sample a single curve was constructed using the mean values for inhibition at each amiloride concentration. To combine the data from more than one skin sample the curves from individual samples were again combined by the same procedure as given above, to give a single concentration-response relationship for either normal or CF epithelia, from which the *K_d* for amiloride was estimated. However pooled data sets take no account of variance between or within cultures derived from different skin samples.

For statistical analysis the model was linearized using logit response and log concentration transformations. Analysis of covariance was performed with a mixed effects linear additive model using PROG GLM from the SAS statistical analysis system (SAS Institute, Inc., North Carolina, U.S.A.). The significance of the difference in *K_d* between normal and CF tissues was analysed using an *F*-ratio test. Statistical assumptions of independent normally distributed error terms were checked using standard diagnostic procedures (Draper & Smith, 1981).

To test whether the *K_d* for amiloride was different when the cultures were bathed in either normal KHS or in low sodium-containing KHS an unpaired Student's *t*-test was used. A *P* value of less than 0.05 was considered significant.

Solutions

The Krebs-Henseleit solution (KHS) used in these experiments had the following composition (mM), NaCl 117, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 11.1. This solution had a pH of 7.4 when gassed with 95% O₂-5% CO₂ at 37°C. In chloride/bicarbonate free solution NaCl, KCl and CaCl₂ were replaced with sodium gluconate 117, potassium gluconate 4.7 and CaSO₄ 2.5. Hepes-Tris 10, adjusted to pH 7.4 was used to replace NaHCO₃, and the solution was gassed with 100% O₂. In sodium free solution *N*-methyl-D-glucamine (NMDG) 117, adjusted to pH 7.4 with HCl was substituted for NaCl, while Hepes-Tris 10 was used to replace NaHCO₃. Substituted KHS solutions were checked for tonicity with a freezing point osmometer and tonicity corrected when necessary by addition of sucrose. Small aliquots of isotonic NaCl were added to sodium free solution to produce graded increases in sodium concentration.

Low Na KHS contained 10mM Na as NaHCO₃ and was gassed with 95% O₂-5% CO₂. The composition was (mM) KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 10, NMDG 117, KH₂PO₄ 1.2 and sucrose 33. When using modified KHS solutions compensation for fluid resistance was made in that solution. Modified KHS solutions were always used symmetrically, i.e. both sides of the tissue were bathed in identical solutions.

Results

Epithelial cultures derived from whole glands, subcultured for 5–8 days on Matrigel-coated filters had the following basal characteristics. For normal tissues prepared from skins of 12 subjects the values were SCC 16.8 ± 1.8 µA cm⁻² (*n* = 49), transepithelial resistance (*R*) 99.2 ± 10.7 Ω cm² (*n* = 49), and transepithelial potential difference (PD) -1.7 ± 0.3 mV (*n* = 49). The corresponding values derived from tissues from four CF patients were for SCC 28.5 ± 3.2 µA cm⁻² (*n* = 18), *R* = 92.2 ± 19.4 Ω cm² (*n* = 18), and PD -2.0 ± 0.4 mV (*n* = 18). There were no significant differences between the two groups with regard to these parameters. While the electrophysiological parameters were relatively constant within a batch of cultures from the same

skin there was wider variation between batches. It might be thought that the SCC, for instance, in controls and CF epithelia was significantly different; this was not so when an analysis based upon the mean batch values was used.

Amiloride concentration-response curves

Two concentration-response curves for amiloride, one for a normal and one for a CF epithelium are shown in Figure 1. The fitted data showed that the concentration causing 50% inhibition of SCC (K_d) was $0.52 \mu\text{M}$ in the normal and $1.05 \mu\text{M}$ in the CF epithelium. The effects of amiloride were reversible upon washing, but here only a single-concentration response curve was obtained for each tissue. For each of the two curves shown in Figure 1 twelve concentrations of amiloride were added. The first (1 nM) and the last ($42.5 \mu\text{M}$) had no effect on SCC, thus defining the threshold and maximally effective concentration range. In neither experiment was the SCC reduced exactly to zero, there being a residual current of some $0.25 \mu\text{A}$. As discussed previously (Brayden *et al.*, 1988) the relatively low potential and resistance of the cultures only enables the position of zero SCC to be estimated to within $0.5 \mu\text{A}$. Notwithstanding, the value of the amiloride-sensitive SCC can be measured with high accuracy.

The data from tissues from six normal patients and from four CF patients were combined as described in the Methods to give two pooled log-concentration-response curves, as shown in Figure 2. The fitted values of K_d and Hill slope (n) for the curves were $0.64 \mu\text{M}$ and 0.88 for normals and $1.07 \mu\text{M}$ and 0.81 for CF respectively. The result of the analysis of covariance showed

Figure 1 Typical cumulative concentration-response curves in sweat gland epithelia from a control and CF patient. Cumulative amiloride concentrations were added every 5 min to produce bath concentrations of (nM) 1, 11, 62, 160, 417, 910 (μM) 1.17, 1.70, 6.80, 12.00, 22.00, 42.50. No inhibition was observed to either the lowest or highest concentration. Horizontal lines indicate zero SCC.

Figure 2 Concentration-response curves for amiloride in sweat gland cultures from normal and CF tissues. The curve for normal tissues (left hand) was derived from 14 cultures in tissues from six patients, the CF curve (right hand) from 11 cultures in tissues from four patients. The curves were derived as described under Methods. Inset shows the equivalent circuit discussed in the text.

that the intercept on the logit axis was different for normals and CF, i.e. the K_d is significantly enhanced in CF. The F ratio with 1, 149 degrees of freedom was 8.66 ($P < 0.02$).

In a few experiments concentration-response curves to amiloride were obtained in a solution in which the sodium concentration was reduced to 10 mM and compared with those obtained in normal KHS solution. Cultures derived from the same skin sample were bathed either in normal KHS or low sodium KHS and cumulative additions of amiloride made. With normal epithelia the fitted K_d of amiloride shifted from $0.71 \mu\text{M}$ in high sodium solution to $0.29 \mu\text{M}$ in low sodium solution (Figure 3a). In CF epithelia the K_d shifted from $1.12 \mu\text{M}$ in high sodium to $0.28 \mu\text{M}$ in low sodium (Figure 3b), thus in the low sodium conditions the K_d values for amiloride were similar in normal and CF tissues, but the shift caused by lowering sodium was greater in CF cultures.

Table 1 summarizes the K_d values for amiloride in different conditions. The conclusions are that the K_d in CF is significantly greater than in normals, while lowering the sodium concentration significantly reduces the K_d in both types of tissue.

The effect of chloride/bicarbonate removal on the K_d of amiloride was also examined. Four cultures obtained from a normal subject were

Figure 3 a) Concentration-response curves for amiloride were obtained in sweat gland cultures from normal tissues bathed in either KHS ($\blacktriangle$) or low sodium KHS ($\bullet$). The K_d in KHS was $0.7 \mu\text{M}$ ($n = 3$) and in the low Na KHS was $0.3 \mu\text{M}$ ($n = 2$). These values were significantly different ($P < 0.005$). b) Similar results and conditions for CF cultures. K_d values were $1.1 \mu\text{M}$ in KHS ($n = 4$) ($\blacktriangle$) and $0.3 \mu\text{M}$ ($n = 3$) ($\bullet$) in low sodium KHS. These values too were significantly different ($P < 0.001$).

used; two cultures were bathed in KHS and two in chloride/bicarbonate free solution. In KHS the K_d values were $0.61 \mu\text{M}$ and $0.64 \mu\text{M}$ while in the modified solution the values obtained were 2.48 and $2.34 \mu\text{M}$ (Figure 4). In a similar experiment using three cultures of CF tissue, derived from the same skin sample, K_d values of $1.43 \mu\text{M}$ and $1.06 \mu\text{M}$ were obtained in KHS solution, while in the absence of permeable anions a value of $2.66 \mu\text{M}$ was found.

Thus far there have been no reports describing the relationship between sodium absorption and sodium concentration using perfused sweat ducts. Although the currents in cultured epithelia

Figure 4 Concentration-response curves for amiloride in four sweat gland cultures from a normal subject. Two curves were obtained with the tissue bathed in normal KH solution ($\circ$, $\blacktriangle$), whereas the two other curves were obtained in chloride/bicarbonate-free solution ($\blacksquare$, $\bullet$).

are small even at normal sodium concentrations it was possible to measure the increases in current caused by the cumulative addition of sodium to cultures originally bathed in a sodium-free (substituted with NMDG) solution. Figure 5 shows the data for six separate experiments with control tissues from two subjects. The form of the relationship is basically hyperbolic reaching a maximal value at around 20 mM Na . All of the current increase was amiloride-sensitive. The concentration of sodium which half saturates the absorptive process is around 4 mM in normal epithelia. In four CF epithelia from two subjects the value rose to around 6 mM , and furthermore the shape of the curve relating to sodium concentration was changed (Figure 5). At all sodium

Figure 5 Percentage increase in SCC vs sodium concentration in sweat gland epithelia. Means and standard errors are given for six measurements ($\bullet$) in normal tissues and four measurements for CF tissues ($\blacksquare$).

Table 1 K_d values in normal and CF epithelia derived from whole glands

		Conditions	K_d (μM)
A.	Tissues bathed in KHS	Normal	0.64 ± 0.02 (6)
		CF	1.07 ± 0.04 (4)*
B.	Normal tissues	Bathed in KHS	0.71 ± 0.04 (3)
		Bathed in Low Na	0.29 ± 0.03 (2)**
C.	CF tissues	Bathed in KHS	1.12 ± 0.06 (4)
		Bathed in Low Na	0.28 ± 0.02 (3)***

In A the numbers in parentheses refer to the number of normal and CF patients studied. In B and C the data refer to a single normal and a single CF patient. The numbers in parentheses indicate the number of tissues examined. Asterisks indicate a significance of $P < 0.02$ (ANOVA)*, $P < 0.005$ (Student's *t*-test)** and $P < 0.001$ (Student's *t*-test)***.

concentrations up to 10 mM the percentage maximal amiloride sensitive short current (ASCC) was significantly less in CF tissues than in corresponding control tissues ($P < 0.05$).

Discussion

There was no significant difference in the transepithelial potentials found for cultures prepared from normal and CF tissues. This is, at first, surprising as it is known that apical chloride channels are non-functional in CF (Welsh & Leidtke, 1986) which expresses itself as an increased transepithelial potential in both the sweat gland (Quinton, 1986) and airway epithelia. Indeed, in airways this increased potential can be sensed as an increased nasal potential in CF patients (Knowles *et al.*, 1981). However, minor defects in the cultured epithelial sheets, such as minor, non-confluent areas would serve to shunt the transepithelial potential. This does not mean, however, that the apical membrane defect in chloride permeability is not present. It may be thought that the transepithelial resistance may give an indication of the confluent nature of the altered epithelium. Cable analysis of microperfused ducts gave a value for transepithelial resistance of $10 \Omega \text{ cm}^2$, due to an excessively high transcellular conductance (Bijman & Fromter, 1986) while our resistances are some ten-fold greater. In contrast Pedersen (1984) found resistances of $400\text{--}1000 \Omega \text{ cm}^2$ for cultured sweat ducts. However in culture sweat gland epithelia develop multilayers, some of which consist of dead cells (Brayden *et al.*, 1988). These features, together with the unknown interaction between cells and the Matrigel coated

Millipore filter probably make conductance measurements in whole cultured epithelia of little value.

Nevertheless, occasional cultured epithelia from this study have had transepithelial potentials of up to 9 mV (apical negative) for normal tissues, a value consistent with the value in intact glands (Bijman & Quinton, 1984) indicating that true transepithelial potentials are possible. The most likely reason, therefore, for the low transepithelial potentials remains as the presence of micro-subconfluent regions in the epithelium.

In this work we have discovered some significant changes in the sensitivity to amiloride in epithelia derived from CF patients compared with normals. We consider this a reflection of the altered chloride conductance of the apical membrane, characteristic of CF. The rest of the discussion explores this hypothesis and considers a variety of possible mechanisms.

Precise measurement of macroscopic K_d values for amiloride, in cultured human sweat gland epithelia, shows that in CF tissues the value is approximately twice that of normal tissues. An analysis of variance has shown these values to be significantly different. The mechanism which underlies this difference is of interest, particularly if it relates to the low chloride conductance characteristic of CF epithelia. There are several possible mechanisms for the recorded effect, as follows. Amiloride sensitive, epithelial sodium channels (Cuthbert & Fanelli, 1978) may be subtly modified in CF. There is no published evidence for this hypothesis. As CF is a single gene defect, it implies a control mechanism is affected which interacts with both Na^+ and Cl^- channels. In airway epithelia from CF subjects sodium transport is enhanced two-fold

(Boucher *et al.*, 1986) but this phenomenon has not been reported for sweat glands. In airways increased sodium transport will contribute to the transepithelial potential, but it has yet to be established whether this is due to an increased number of channels or of altered sodium channel characteristics. If the latter, changes in the binding characteristics of amiloride might be expected.

In many electrogenic sodium absorbing epithelia there is an apparent competition between sodium and amiloride, the K_d for amiloride decreasing with sodium concentration (Cuthbert & Shum, 1974). Furthermore specific amiloride binding in epithelia is inhibited by sodium (Aceves & Cuthbert, 1979) and indeed it is proposed that sodium ions have weak amiloride like activity, thus explaining sodium self inhibition (Zeiske & Lindemann, 1974). Single channel currents apparently do not saturate with increasing sodium concentration (Van Driessche & Lindemann, 1979), the curvilinear relationship of SCC to sodium concentration being due to increasing blockage of sodium channels by sodium. A second possible mechanism is that, in CF, sodium self-inhibition is modified such that sodium ions are a more effective antagonist of amiloride. It is shown here that there is an apparent amiloride-Na competition in cultured sweat glands, as reducing the sodium concentration from 142 mM to 10 mM results in a parallel shift in the concentration-response curve for amiloride. However the left-ward shift on reducing sodium concentration was two-fold in normals and four-fold in CF epithelia (Table 1), giving some credence to the idea that sodium more effectively antagonises amiloride in CF. Furthermore we have noted subtle changes in the shape of the curve relating sodium concentration to SCC in CF tissues.

Another possibility is that the action of amiloride is potential sensitive, i.e. the drug molecule senses some part of the apical membrane potential, which is different in CF and control epithelia. As there are no studies of the apical membrane potential in short circuited sweat duct epithelia we have approached the problem in relation to a simplified electrical equivalent circuit. The circuit is essentially similar to the pair of circuits proposed by Reddy & Quinton (1987) in which there is both a paracellular and transcellular shunt pathway (Figure 2). In this circuit E_1 , R_1 and R_3 represent the apical sodium conductive pathway (E_1 , R_1) shunted by a chloride conductance (R_3) while E_2 , R_2 represents the basolateral potassium conductive pathway shunted by a chloride conductance. R_s represents a paracellular shunt pathway and the third arm short circuits V_{ab} . The apical

membrane potential, V_{ab} , is given by

$$V_{ab} = E_1 - \frac{E_1}{R_1 + \frac{R_2 R_3}{R_2 + R_3}} \cdot R_1 \\ - \frac{E_2}{R_2 + \frac{R_1 R_3}{R_1 + R_3}} \cdot \frac{R_3}{R_1 + R_3} \cdot R_1$$

which simplifies to

$$V_{ab} = \frac{E_1 R_2 - E_2 R_1}{\frac{R_1 R_2}{R_3} + R_1 + R_2}$$

It is known that Cl^- ions move passively across sweat duct epithelia through a large selective shunt pathway (Quinton, 1986). It is also known that chloride channels in the apical membrane are affected in CF, although whether the paracellular pathway is affected is unknown (Welsh & Liedtke, 1986). Thus a simplistic view of the effect of CF or chloride/bicarbonate replacement upon V_{ab} is to consider the effect of R_3 becoming very large. Clearly V_{ab} is larger when $R_3 \rightarrow \infty$, i.e. the potential inside the cell is relatively more positive. This is not inconsistent with the values of open circuit potentials across the apical membrane of ducts. V_{ab} changed from -27 mV to $+11$ mV when gluconate replaced chloride (Reddy & Quinton, 1987) while in CF V_{ab} has a value of $+36$ mV (Reddy & Quinton, 1986). The active form of amiloride is the positively charged guanidinium ion (Cuthbert, 1976) interacting with an anionic group with a pK_a of 5. If this acid grouping is embedded within the membrane then it can be expected that its interaction with amiloride will be potential sensitive, being promoted by a membrane field in which the inside of the membrane is negative with respect to the outside. Interestingly in the perfused rabbit salivary duct the K_d for amiloride is changed from $0.3 \mu\text{M}$ to $3.0 \mu\text{M}$ when permeant anions were removed, using measurements of transepithelial conductance as an index (Bijman *et al.*, 1983). Although the change in K_d is larger the result is not inconsistent with the present findings. However the proposal that the altered K_d for amiloride is a consequence of the reduced chloride conductance of the apical membrane may need further consideration. It has been widely canvassed, but not published, that the reported chloride channel defects in CF disappear when experiments are carried out at 37°C rather than room temperature. Neverthe-

less there are clear differences between normal and CF chloride channels which may be emphasised by lowering the temperature. It is noted in this work that when permeant anions are removed, effectively removing apical chloride conductance, the shift in K_d for amiloride is greater than the shift between normals and CF tissues. It would be reasonable to conclude that chloride conductance is impaired rather than abolished in CF. The raised salt content of CF sweat, together with the increased transepithelial potential, is difficult to explain without impaired chloride conductance at body temperature.

Moran *et al.* (1985) investigating the mammalian colon considered that the change in sensitivity to amiloride at different sodium concentrations was due to changes in Vab. However in other systems (Lindemann & Voute, 1976) sodium-amiloride competition was needed to explain the changes in sensitivity with sodium. In these two types of system there was an important difference in relation to estimates of K_{Na} (concentration of sodium which half saturates the transport process). This can be estimated from the relation of [Na] to SCC and from the relation of K_d for amiloride and Na (for discussion see Cuthbert, 1987). In the colon and coprodeum (Bindslev *et al.*, 1982) the two estimates for K_{Na} were very different, while in amphibian skins the values were identical. In the cultured sweat gland values of K_{Na} were 4 mM, estimated from the dependence on [Na], and 80 mM (normal) and 30 mM (CF) calculated from data in Table 1. Thus cultured, human sweat glands behave

similarly to the colon and coprodeum where a potential dependence has been demonstrated and unlike amphibian skin where no effect on K_d for amiloride was found when the apical membrane potential was altered by passing current (Cuthbert & Shum, 1976). This does not rule out the possibility that the shift in the concentration-response curves to amiloride at low sodium concentrations in sweat ducts has a competitive element.

Finally the dependence of SCC on sodium concentration in sweat ducts gives a value for K_{Na} of around 4 mM. If this value is true too *in vivo* it means that, even in normal individuals with low salt concentrations in sweat (*circa* 15 mM), the process is virtually saturated. Yet to prevent excessive salt loss at high sweating rates demands this reabsorptive process be regulated. To do this it seems necessary either to increase the electrochemical gradient for sodium entry or to recruit more entry sites. The former might be achieved by increasing activity of the basolateral pump or increasing K permeability of the basolateral membrane to achieve apical membrane hyperpolarisation. The latter would require recruitment of apical sodium channels and the possibility that ductal absorption might be influenced by neurotransmitters or autacoids affecting these or other processes cannot be ignored.

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