Ion transport in cultured epithelia from human sweat glands: comparison of normal and cystic fibrosis tissues

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- 1 Cultured epithelia derived from whole human sweat glands, isolated secretory coils, isolated reabsorptive ducts and whole glands from cystic fibrosis (CF) subjects have been used to examine drug sensitivity by use of short circuit current recording.
- 2 Short circuit current increases were observed with lysylbradykinin, carbachol and histamine in epithelia of different origins. All responses were due to stimulation of electrogenic sodium absorption, evidenced by the inhibition of these responses by amiloride. The latter also abolished the basal current. The terpenes, thapsigargin and forskolin had no effect on transport.
- 3 The stimulation of a sodium current by agonists was dependent upon calcium, responses being inhibited by lanthanum ions and EGTA. Further A23187 induced a sodium current.
- 4 Pronounced oscillations in the sodium currents were a feature of the responses, implying synchronous, regulated cell activity.
- 5 Forskolin produced a ten fold increase in adenylate cyclase activity. All agonists listed in 2 except forskolin caused an increase in intracellular calcium $[Ca]_i$. $[Ca]_i$ responses in CF cells were not different from those of normal cells, except with thapsigargin where the responses were smaller.
- 6 It is concluded that in culture, cells develop ductal characteristics, whether derived from normal or CF glands, coils or ducts. An increase in $[Ca]_i$ followed by activation of calcium-sensitive potassium channels and apical membrane hyperpolarization may be the major mechanism for increasing sodium influx.

Introduction

The geometry of the human sweat gland makes investigation of transepithelial ion transport difficult. However, human sweat glands can be grown in primary culture, then disaggregated and plated upon pervious supports to form small epithelial sheets. These sheets can be short circuited and the nature of the transported species obtained from ion substitution experiments and use of blocking drugs. Recently data have been obtained with whole human sweat glands (Brayden *et al.*, 1988) and human sweat gland ducts (Pedersen *et al.*, 1987). In a few instances (Brayden *et al.*, 1988) experiments were done with cultures derived from separated sweat gland coils. These coil-derived epithelia demonstrated electrogenic sodium absorption under short circuit current (SCC) conditions *in vitro*, rather than chloride secretion. Indeed they behaved similarly to cultures from whole glands. Furthermore, cultures with duct-like characteristics responded to cholinergic agonists and isoprenaline, although it is not generally considered that salt reabsorption in the duct is under autonomic control. Cultures derived from whole cystic fibrosis (CF) glands also demonstrated electrogenic sodium absorption, but they showed a reduced sensitivity to amiloride compared to controls (Cuthbert *et al.*, 1990). This effect is considered to result from the reduced apical chloride conductance, characteristic of the disease (Welsh & Leidtke, 1986; Frizzell *et al.*, 1986).

This present study explores further features of cultured sweat gland epithelia. The spectrum of agents that promote sodium transport is expanded and additionally the effects of agents on cultures derived from separated coils and ducts is given. Particular attention has been paid to the role of the intracellular messengers $[Ca]_i$ and adenosine 3':5'-cyclic monophosphate (cyclic AMP) in the stimulation of sodium transport. Recently, the gene responsible for CF has been cloned (Rommens *et al.*, 1989). The normal gene apparently codes for a membrane protein of 1480 amino acids with two nucleotide binding folds called CFTR (cystic fibrosis trans-

membrane regulator). In CF a phenylalanine at position 508 is missing. The protein has a close similarity to mdr 1 (p-glycoprotein), the membrane protein responsible for multiple drug resistance although the precise function of CFTR is unknown. It is apparent that more than one cellular function is perturbed in CF. An understanding of the pharmacological responsiveness of CF epithelia may provide clues for possible therapeutic strategies for the disease. In this connection the improved lung function in CF patients receiving nebulised amiloride (Knowles *et al.*, 1990) is of interest.

Methods

Isolation and culture of sweat glands

Non-cauterized skin samples were obtained at surgery from normal and CF patients, and occasionally from punch biopsies. Permission was obtained from Huntingdon Health Authority Ethics Committee. The skin was placed in 10 ml of sterile buffer and sweat glands isolated by the procedure described by Lee *et al.* (1984).

Primary cultures of whole human sweat glands were grown as described previously (Brayden *et al.*, 1988). In some instances primary cultures were grown from secretory coil or from reabsorptive duct. Glands were microdissected as described by Lee *et al.* (1986), briefly by collagenase digestion and recovery in a medium containing bovine serum albumin after which the glands were gently uncoiled. The coil was distinguished on the basis of appearance and a wider diameter and both coil and duct were separated at a point distal from the transitional junction. The transitional junction region was placed in dilute neutral red buffer to check that a diffuse red area (i.e. coil) merged with a thin dark red line (duct) thus validating the integrity of the separated coil.

After two to three weeks the primary cultures were dispersed with trypsin-versene (as described by Brayden *et al.*, 1988) and used to seed small wells closed by Millipore filters (0.2 cm²) coated in Matrigel, using 0.2 to 0.5 × 10⁶ cells per

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well (Cuthbert *et al.*, 1987). Cultured epithelia were ready for use in five to eight days. Measurements of transepithelial potential, transepithelial resistance and short circuit current (SCC) were made as described previously (Cuthbert *et al.*, 1987). Briefly, a WP dual voltage clamp with a facility for fluid resistance compensation was used. After a period for stabilization (circa 20 min) the transepithelial potential was noted after which the monolayers were short-circuited throughout the rest of the experiment. Intermittently the voltage was clamped at ± 1 mV and the current required to do this used to calculate the resistance.

Solutions

The bathing solution used for the electrophysiological work was Krebs-Henseleit which had pH 7.5 at 37°C when bubbled with 95% O₂ and 5% CO₂. The composition of this solution was (mM): NaCl 117, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25 and glucose 11.1. This solution was modified in some experiments. When barium ions were used MgSO₄ was replaced with MgCl₂ and bicarbonate was replaced with HEPES-Tris (10 mM) and NaCl increased to 142 mM. The same was true when lanthanum was used and additionally KH₂PO₄ was omitted and KCl increased to 5.7 mM. Both of these modified solutions were gassed with O₂. To remove ionized calcium from the bathing solution the appropriate amount of EGTA solution, 36 mM was added which had been previously neutralized to pH 7.4 with NaOH. This solution did not alter the tonicity of the medium or its sodium concentration; the final EGTA concentration was 2.5 mM. Readdition of CaCl₂ solution was able to restore the original ionized calcium concentration in the presence of the EGTA. To make low chloride Krebs solution (5.0 mEq l⁻¹) NaCl and KCl were replaced with sodium gluconate and potassium gluconate respectively.

Measurement of [Ca]_i

Intracellular calcium ion concentrations [Ca]_i were measured by Fura 2 fluorescence (Grynkiewicz *et al.*, 1985). After 2–3 weeks in culture, cells were dispersed in trypsin-versene as above, separated by gentle centrifugation and resuspended in the following medium (mM): NaCl 137, KCl 5.4, CaCl₂ 1.0, KH₂PO₄ 0.4, MgSO₄ 0.3, glucose 1.1, HEPES 10, bovine serum albumen 1 mg ml⁻¹ and Fura 2-AM, 2 µM at pH 7.4. The cells were incubated, with gentle shaking, at room temperature for 45 min. Aliquots (2 ml) of the suspension, containing 2–3 × 10⁶ cells, were rapidly centrifuged and washed in buffer without Fura 2-AM, and the cells transferred to a cuvette maintained at 37°C and stirred. Fluorescence was measured in a Perkin-Elmer LS5B luminescence spectrometer at 510 nm with excitation at 340 nm and 380 nm. After subtraction of the autofluorescence the ratio of the intensities at 340 nm and 380 nm were computed every 5 s. [Ca]_i was computed from the formula

$$[\text{Ca}]_i = K_d \times \frac{R - R_{\min}}{R_{\max} - R} \times S$$

where $K_d = 224$ nM, R is the ratio of intensity at 340 nm and 380 nm. $R_{\max}$ is the same ratio in the presence of excess calcium (12 mM) and $R_{\min}$ the ratio in the absence of calcium (10 mM EGTA). S is the ratio of the fluorescence at 380 nm in calcium-free and excess calcium conditions. When [Ca]_i was measured continuously without drug addition there was a slow increase in basal level. This was due to Fura-2 leakage from the cells since after centrifugation the supernatant gave a fluorescence insensitive to digitonin but abolished by Mn. Consequently increases caused by agonists were calculated from the immediately preceding basal value.

Adenylate cyclase assay

Whole sweat glands were cultured as given above and scraped from the plastic surface after 2–3 weeks. To measure adenylate

cyclase activity, sweat gland cells were homogenized in a buffer containing (mM): Tris-HCl 25, MgCl₂ 5 and creatine phosphate 20. Aliquots of this suspension were added to the reaction mixture containing (mM): Tris HCl 25, MgCl₂ 5, creatine phosphate 20, cyclic AMP 1, GTP 0.5, ATP 1, isobutylmethylxanthine (IBMX) 0.2, creatine phosphokinase 100 µg ml⁻¹ and α³²P-ATP (5 × 10⁶ c.p.m. per assay). The reaction was started by adding the homogenate and stopped by adding sodium dodecylsulphate. The reaction was carried out at 30°C and pH 7.5 for 15 min. To separate cyclic AMP from ATP the reaction mixtures were run over Dowex 50 columns onto alumina columns and the cyclic AMP eluted with imidazole buffer (0.1 M). Appropriate blanks (no reaction mixture or no homogenate) and controls (e.g. linearity between volume of homogenate and activity) were carried out. Protein concentrations of the homogenates were measured by the method of Lowry *et al.* (1951). Results were expressed as pmol cyclic AMP mg⁻¹ protein h⁻¹.

Student's *t* test (unpaired) was used to test for significance, with $P < 0.05$ considered significant.

Drugs and chemicals

Forskolin, Fura 2-AM and ionomycin were obtained from Calbiochem, benzimidazole guanidine from Pfaltz & Bauer Inc., cimetidine from Aldrich Chemical Co Ltd. and the following drugs from Sigma Chemical Co. Ltd.: A23187, piroxicam, atropine, carbachol, dibutyl cyclic AMP, dibutyl cyclic GMP, histamine, mepyramine, isoprenaline, lysylbradykinin, neomycin sulphate. Peptides were from Peninsula Laboratories Inc. All other chemicals were of reagent grade. Thapsigargin was a gift from O. Thastrup.

In general, drugs were dissolved in distilled water at concentrations such that only minute volumes were added to preparations. An exception was neomycin which was dissolved in KHS because of the large volume of solution required to achieve the desired concentration.

Results

Spectrum of agonist sensitivity in normal and CF epithelia

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 values were SCC, $16.8 \pm 1.8 \mu\text{A cm}^{-2}$ ($n = 49$), transepithelial resistance $99.2 \pm 10.7 \Omega \text{ cm}^2$ ($n = 49$) and for transepithelial potential (apical side negative), $1.7 \pm 0.3 \text{ mV}$ ($n = 49$). Note that n gives the total number of epithelia examined. The comparable values derived from tissues from four CF patients were SCC = $28.5 \pm 3.2 \mu\text{A cm}^{-2}$ ($n = 18$), $R = 92.2 \pm 19.4 \Omega \text{ cm}^2$ ($n = 18$) and PD = $2.0 \pm 0.4 \text{ mV}$ ($n = 18$).

In a few experiments epithelial sheets were prepared from microdissected secretory coils or reabsorptive ducts from normal glands. The basal transporting characteristics of these tissues fell within a similar range to those above and were as follows. For secretory coil epithelia values were SCC = $24.3 \pm 3.2 \mu\text{A cm}^{-2}$ ($n = 7$), PD = $2.3 \pm 0.6 \text{ mV}$ ($n = 7$) and $R = 70.0 \pm 11.8 \Omega \text{ cm}^2$ ($n = 7$). Many reabsorptive ducts were required to produce enough cells to seed filters and only two confluent monolayers prepared exclusively from ducts were obtained. Mean values were $30 \mu\text{A cm}^{-2}$, 0.7 mV and $28 \Omega \text{ cm}^2$ respectively for SCC, PD and R .

One aim of this investigation was to explore the spectrum of agonists that could affect SCC in these epithelia. While the actions of some agonists have been described by us before (Brayden *et al.*, 1988) their actions have not been described on cultures derived from separated coils and ducts. Supramaximal concentrations of agonists have been used as it was not possible to determine concentration-response relationships in these delicate structures, except by cumulative addi-

tion. However, marked desensitization precluded this option. It is also of interest to record agonists that were ineffective in increasing SCC, especially if they produce secondary changes in intracellular messengers. The bulk of this information is given in Table 1 which should be studied alongside Figures 1–4 and the statements below. Figure 1 shows results for epithelia cultured from whole sweat glands. The oscillations in SCC following histamine are characteristic of all agonists investigated. However oscillations were not seen invariably and the frequency of occurrence is given in Table 1. Oscillations were not exclusive to normal epithelia, occurring with a similar frequency in epithelia cultured from whole CF sweat glands. Figure 2 shows oscillations in CF epithelia following lysylbradykinin (LBK) and carbachol. Note both the agonist-induced current and oscillations, but not the basal current, were immediately curtailed by atropine following carbachol. The effect of atropine was different from that of amiloride (Figure 1) which abolished both the oscillations and basal current. Figure 3 is a tracing produced with an epithelium derived from secretory coils only. This was chosen to illustrate responses almost free of oscillations, although coil cultures could also show these. Cultured epithelia derived from reabsorptive ducts also showed sensitivity to LBK and carbachol and are also capable of oscillatory behaviour (Figure 4). The effects of other agonists not illustrated are given in Table 1. Some clues as to mechanisms were obtained by use of inhibitors. For example, piroxicam, $5 \mu\text{M}$, failed to block the effects of LBK, suggesting the latter's effects were not due to prostaglandin formation. Atropine, 10 nM blocked the effects of carbachol or curtailed its effect if given after the agonist (Figures 2, 3) indicating an action at muscarinic receptors. Mepyramine, 100 nM , but not cimetidine, $1 \mu\text{M}$, blocked the effects of histamine indicating the presence of H_1 -receptors.

Figure 1 Illustrates the effects of histamine (Hist, $10 \mu\text{M}$) on SCC in epithelia cultured from normal whole glands. With histamine the SCC response is sustained compared to that for isoprenaline (Iso, $10 \mu\text{M}$). In (b) amiloride ($10 \mu\text{M}$) was added before either agonist, while the reverse was true for (a). Both preparations were from the same batch. Horizontal lines indicate zero SCC and calibrations are the same for both traces.

Table 1 Short circuit current responses ($\mu\text{A cm}^{-2}$) in cultures of whole sweat gland epithelia: comparison of normal and cystic fibrosis (CF) tissues

Agonist	Normal	CF
Lysylbradykinin ($0.1 \mu\text{M}$)	15.7 ± 1.7 (14/53)	11.2 ± 1.3 (4/10)
Carbachol ($10 \mu\text{M}$)	15.1 ± 1.8 (10/32)	14.3 ± 1.1 (4/5)
Histamine ($10 \mu\text{M}$)	13.0 ± 2.2 (7/18)	13.3 ± 3.7 (3/3)
Isoprenaline ($10 \mu\text{M}$)	6.2 ± 1.3 (2/11)	6.6 ± 1.6 (0/3)
A23187 ($1 \mu\text{M}$)	5.3 ± 0.9 (2/6)	4.2 (2/2)
Thapsigargin (170 nM)	-0.3 ± 0.4 (0/5)	1.6 ± 0.5 (0/4)
Ionomycin ($1 \mu\text{M}$)	11.3 ± 2.3 (0/3)	—
Adenosine triphosphate ($1 \mu\text{M}$)	—	33.8 (0/1)

Numbers in parentheses indicate number of separate preparations examined, the first of two figures indicating the number showing oscillations in response to particular agonists.

Figure 2 SCC responses of a whole gland culture made from cystic fibrosis glands. Notice that time scale was temporarily expanded to show the contour of the oscillations. Lysylbradykinin (LBK) $0.1 \mu\text{M}$, carbachol (CCh) $10 \mu\text{M}$, and atropine (Atr) $0.1 \mu\text{M}$ were added as indicated. Horizontal line indicates zero SCC.

Figure 3 SCC responses in a culture derived from secretory coils from normal glands. Responses to lysylbradykinin (LBK) ($0.1 \mu\text{M}$), carbachol (CCh) ($10 \mu\text{M}$), histamine (Hist) ($10 \mu\text{M}$) and isoprenaline (Iso) ($10 \mu\text{M}$) are shown. Atropine (Atr) (10 nM) abolished the response to CCh. Note only minor oscillations are seen following LBK. Horizontal line indicates zero SCC.

Figure 4 SCC responses in a culture produced from reabsorptive ducts from normal glands. Concentrations of drugs were lysylbradykinin (LBK, $0.1 \mu\text{M}$), carbachol (CCh, $10 \mu\text{M}$) and amiloride (Amil, $10 \mu\text{M}$). Horizontal line indicates zero SCC.

A number of agents had no effect on SCC. For example, benzimidazole guanidine, which promotes sodium movement through amiloride-sensitive channels, caused minor SCC inhibition like amiloride. This is surprising as amiloride-sensitive sodium channels are clearly present (see later). It is shown later that an intact adenylate-cyclase system is present in sweat gland epithelia, yet no SCC responses were obtained in response to cyclic AMP, cyclic GMP (both as dibutyl derivative at $0.1 \mu\text{M}$) or forskolin, $10 \mu\text{M}$. No effect of the neuropeptides vasoactive intestinal polypeptide, neuropeptide Y, arginine vasopressin or atrial natriuretic peptide were seen. There are reasons to expect the sweat gland epithelium would be sensitive to these agents (see Discussion). We did not find any agents which had exclusive actions on normal or CF epithelia. Finally the effects of the terpene thapsigargin are of particular interest since it is shown later that this agent has a profound effect on $[\text{Ca}]_i$ but is almost without effect on SCC (Table 1).

Nature of the transported ion

It is already apparent from Figures 1, 3 and 4 that the basal SCC or the additional current induced by agonists is removed by amiloride, $10 \mu\text{M}$. At this concentration amiloride is specific for apical epithelial sodium channels. It may therefore be concluded that all the SCC is due to electrogenic sodium absorption, both in normal and CF tissues, and in secretory coil and duct derived epithelia. Electrogenic anion secretion might have been expected in the epithelia derived from secretory coils. Conditions were manipulated to increase the possibility of seeing a minor secretory response. Amiloride was added to block sodium absorption and to cause apical membrane hyperpolarization, a condition favouring chloride exit through the apical face. Secondly, the apical chloride concentration was reduced to 5.0 mEq l^{-1} , replacing with gluconate, to impose a favourable chloride gradient for secretion (Willumsen & Boucher, 1989). Small responses to several agonists were seen, no bigger than the residual ones seen in normal solution in the presence of amiloride.

To examine further if the responses to agonists were due to sodium absorption experiments were carried out in which agonists were added either before or after amiloride, $10 \mu\text{M}$, with statistical comparison of the responses obtained. This was done with a batch of CF cultures and the agonists LBK and carbachol, in order to compare with similar data obtained in normal tissues previously (Brayden *et al.*, 1988). In the second experiment a batch of normal cultures were used to probe the effect of amiloride on the responses to isoprenaline and histamine. The data are summarized in Table 2. While there is a highly significant reduction in the size of the responses there were small residual agonist effects. These were too small to investigate systematically, but it is possible they may have a different ionic basis to those inhibited by amiloride.

Table 2 Effects of amiloride on responses to various agonists in cultures of whole normal glands and cystic fibrosis glands

	Before amiloride	After amiloride	P
<i>Normal</i>			
Isoprenaline	7.9 ± 2.5 (5)	0.8 ± 0.5 (5)	<0.025
Histamine	10.8 ± 3.8 (4)	1.1 ± 0.6 (4)	<0.025
<i>Cystic fibrosis</i>			
LBK	18.0 ± 3.2 (4)	2.4 ± 3.3 (5)	<0.001
Carbachol	17.9 ± 3.3 (5)	3.3 ± 0.7 (5)	<0.001

All values are of SCC ($\mu\text{A cm}^{-2}$). Amiloride (apical application) was used at $10 \mu\text{M}$. Agonists are applied basolaterally at the following concentrations; isoprenaline $10 \mu\text{M}$, histamine $10 \mu\text{M}$, lysylbradykinin (LBK), $0.1 \mu\text{M}$ and carbachol $10 \mu\text{M}$. An unpaired Student's *t* test was used to test for significance of the effect of amiloride.

Second messenger systems involved in the SCC responses

Adenylate cyclase activity in homogenates of normal cultured sweat gland cells showed a ten fold increase in response to forskolin, indicating the presence of the enzyme (Table 3). LBK can stimulate adenylate cyclase activity indirectly through the intermediary of prostaglandins. However, no stimulation of activity was found with LBK, as expected since responses to LBK were not modified by piroxicam. While isoprenaline caused a doubling of activity this increase was not significant. The presence of β -adrenoceptors coupled to adenylate cyclase in these cells is therefore unproven.

In contrast to the unlikely role for cyclic AMP in transport in these *in vitro* epithelia, a strong case can be made for the involvement of intracellular calcium ($[\text{Ca}]_i$). First, lanthanum ions added during the plateau of an agonist response immediately removes the added SCC without severely affecting the basal SCC (Figure 5a). Added before an agonist lanthanum ions had no immediate effect on basal SCC. This might suggest that there is a requirement for continued calcium influx during the plateau response, while the basal SCC is not so dependent. Secondly, A23187, a calcium ionophore was able to stimulate SCC and oscillations in cultured sweat gland epithelia. Further, as with lanthanum, removal of calcium influx by chelation with EGTA removed the stimulated component of the current while leaving the basal largely intact. The effect of EGTA was reversible by adding calcium ions (Figure 5b), and had no immediate effect on basal SCC when added alone.

In contrast to the effects of lanthanum ions, barium ions affected the basal SCC. Barium acts as a weak blocker of calcium-sensitive potassium channels present in sweat gland cells (Henderson *et al.*, 1990). Barium ($5 \mu\text{M}$) applied basolaterally caused SCC to decrease by $54 \pm 5\%$ ($n = 6$) in epithelia from normal glands and by 35% (mean of 2) in CF epithelia. Responses to the agonists LBK, carbachol and histamine were not attenuated by barium (Figure 6). In 8 experiments made

Table 3 Adenylate cyclase activity in cultured normal sweat gland cells

	Cyclic AMP ($\text{pmol mg}^{-1} \text{ h}^{-1}$)
Control (3)	98.6 ± 31.6
Forskolin $10 \mu\text{M}$ (3)	$1024.3 \pm 210.0^*$
LBK, $0.1 \mu\text{M}$ (3)	116.0 ± 48.1
Isoprenaline, $10 \mu\text{M}$ (2)	229.0 ± 63.6

* $P < 0.01$, unpaired *t* test. Numbers in parentheses indicate number of observations of cultures from separate subjects. LBK: lysylbradykinin.

Figure 5 (a) Shows the effect of lanthanum ions (1 mM) applied basolaterally on the response to carbachol (CCh, $10 \mu\text{M}$). (b) Effect of A23187 ($1 \mu\text{M}$) on SCC after which external calcium ions were removed then replaced by addition of EGTA and CaCl_2 respectively. Finally amiloride ($10 \mu\text{M}$) was added. Both preparations were cultured from whole cystic fibrosis glands. Horizontal lines indicate zero SCC.

Figure 6 Responses of two normal epithelia from the same batch derived from whole glands. Lysylbradykinin (LBK, $0.1 \mu\text{M}$), carbachol (CCh, $10 \mu\text{M}$) and histamine (Hist, $10 \mu\text{M}$) were added in one instance after and in the other before barium ions (5 mM) were added to the basolateral side. Amiloride, $10 \mu\text{M}$, was added at the end of each experiment, apically. Horizontal lines indicate zero SCC and calibration applies to both traces.

with two batches of epithelia the effects of LBK, $0.1 \mu\text{M}$ given before or after barium were respectively $4.7 \pm 1.5 \mu\text{A cm}^{-2}$ and $6.2 \pm 3.6 \mu\text{A cm}^{-2}$, thus barium ions were not an effective blocker of kinin action. Interestingly apically applied barium ions did not affect the basal current indicating a probable effect on basolateral K-channels.

Agonist effects on intracellular calcium $[\text{Ca}]_i$

To confirm the presumed effects of agonists on $[\text{Ca}]_i$ direct measurements with fura-2 fluorescence were made with cell suspensions. The aims were two fold; first to discover if all agonists could increase $[\text{Ca}]_i$ and secondly to establish for given agonists, namely LBK and carbachol, whether the responses were different in extent in normal and CF tissues.

The experimental design posed some problems because of the paucity of experimental material. It was necessary to add more than one agonist to the cell suspension in the cuvette to conserve cells. However, it is known in some situations (Pickles, unpublished observations) that when agonists are given close upon one another the response to the second can be impaired, presumably because both release from the same intracellular store. Consequently agonists were added to cell suspensions at least 5 min apart. Figure 7 illustrates typical traces for single suspensions of sweat gland cells from normal and CF tissues. Reference to Table 4 shows the results of 17 experiments, 10 with CF cells and 7 with normal cells. In the experiments with CF cells, carbachol and LBK were given alternately either first or second. While, in general, when an agonist was given second the responses, both peak heights and plateau values, were smaller they were not significantly so compared with the first responses. This analysis was not carried out for the results with control cells as carbachol was

Figure 7 Illustrative effects of carbachol (CCh, $10 \mu\text{M}$), lysylbradykinin (LBK, $0.1 \mu\text{M}$) and thapsigargin (Tg, 170 nM) on $[\text{Ca}]_i$ measured in suspensions of normal (a) and cystic fibrosis (b) sweat gland cells. Peak heights and plateau values given in Table 4 were measured from the immediately pre-existing value before the agonist was added.

only given second in two of the six measurements, and yielded the largest responses. Table 4 reveals several interesting features as follows: (a) Basal $[\text{Ca}]_i$ values in normal and CF cells are identical; (b) there are no significant differences in the responses to LBK or carbachol in normal and CF cells when comparing either the peak responses or the maintained plateau levels, furthermore responses to LBK are less well sustained than those to carbachol, and (c) there appears to be an enhanced response to thapsigargin in normal cells compared to CF. Two other agonists, namely ATP and histamine, are shown to increase $[\text{Ca}]_i$ in both normal and CF cells. The data here are limited and no comment can be made about comparability, only capability.

A likely mechanism for the increase in $[\text{Ca}]_i$ following LBK is via activation of the phospholipase C system with the consequent production of inositol phosphates and diacylglycerol (Shayman & Morrison, 1985). To test this hypothesis the phospholipase inhibitor neomycin was used. In four normal whole gland cultures the response to LBK ($0.1 \mu\text{M}$) was $2.11 \pm 0.19 \text{ nEq}$ (in these experiments the response was mea-

Table 4 Intracellular calcium increases in response to lysylbradykinin (LBK), carbachol and thapsigargin

Agonist	Control cells			CF cells		
	Peak	Plateau	n	Peak	Plateau	n
Carbachol $10 \mu\text{M}$				172 ± 39 (F)	147 ± 37 (F)	5
				114 ± 27 (S)	85 ± 18 (S)	4
				146 ± 26 (T)	119 ± 23 (T)	9
LBK $0.1 \mu\text{M}$	161 ± 37	101 ± 21	6	232 ± 67 (F)	45 ± 14 (F)	5
				182 ± 18 (S)	70 ± 7 (S)	3
	184 ± 109	41 ± 18	6	214 ± 41 (T)	54 ± 10 (T)	8
Thapsigargin 160 nM	531 ± 110	252 ± 40	4	260 ± 25	156 ± 32	7
				$P < 0.02$	$0.05 < P < 0.1$	
ATP 1.0 mM	216	101	3	342	185	1
Histamine $10 \mu\text{M}$	107	53	2	118	0	1
Basal	189 ± 13	—	7	195 ± 8	—	10

Note: F, S and T refer respectively to first responses, second responses and total first and second responses. Statistical comparisons are to the relevant control value. All values are given as nM, basal values are the values immediately after the cell suspensions are placed in the cuvette and have achieved 37°C .

sured as total charge transfer to account for both the size and duration of the responses). In four other preparations from the same batch, exposed to neomycin 15 mM, the response to LBK was reduced significantly to 0.70 ± 0.16 nEq ($P < 0.005$). Neomycin had a transient effect on SCC which returned to normal before the experiment commenced. This effect was not due to the vehicle which had no effect alone.

Discussion

Reported for the first time in this study is the effect of a variety of agonists on transepithelial ion transport in cultured epithelia from normal and CF whole sweat glands and from cultures derived from separated coils and ducts of whole sweat glands.

In general, the transepithelial potentials were lower than expected from measurements in whole glands (10 mV, lumen negative in normals and 70 mV in CF glands; Bijman & Quinton, 1984; Quinton, 1983; 1986; Bijman & Fromter, 1986) which is probably due to structural imperfections in the monolayers, allowing additional shunt pathways to reduce the potential. It was found (data not shown) that substitution of impermeant anions, such as gluconate, did not substantially alter the potentials indicating pathways permeant to these large anions must exist.

Cable analysis in microperfused ducts gave a value for transepithelial resistance of $10 \Omega \text{ cm}^2$ due it is claimed to high chloride conductance of the ductal cell membranes rather than to leaky tight junctions (Bijman & Fromter, 1986). Our resistance values are up to an order of magnitude greater, which may result from the multilayered structures which develop *in vitro* (Brayden *et al.*, 1988).

We have increased substantially the number of different receptor types now known to be located in the membranes of cultured human sweat glands, both normal and CF. These include histamine H_1 receptors in normal glands, receptors for LBK, histamine, carbachol (muscarinic) and isoprenaline in CF cells. Further, muscarinic receptors and those for LBK are found in cultures derived entirely from secretory coils or from absorptive ducts. Table 1 shows that the responses to LBK, carbachol, histamine and isoprenaline are remarkably similar in normal and CF tissues. Again all four agonists can produce SCC oscillations, but this is not invariably so. These oscillations must indicate that the transporting activity of individual cells can be synchronized, presumably by intercellular communication, although the signal oscillator generating this activity is unknown.

It has been shown here that the effects of histamine and isoprenaline in normal cells and LBK and carbachol in CF cells are significantly inhibited by amiloride, $10 \mu\text{M}$. The unique specificity of amiloride used at this concentration (Cuthbert & Shum, 1974) is strong evidence that these agents stimulate electrogenic sodium absorption. As the basal SCC is also abolished by amiloride at this concentration it is likely that the only substantial activity shown by any of the cultures we have examined is sodium absorption. The failure to see chloride secretion in coil cultures is puzzling as this is the normal function of this section of the gland. Attempts to enhance the small residual effects seen after amiloride by imposing a favourable chloride gradient were unsuccessful. Unfortunately the residual responses were too small to investigate systematically.

Considering the epithelia are sodium absorbing, it is of interest that arginine vasopressin and atrial natriuretic peptide which can stimulate and inhibit respectively electrogenic sodium absorption in other tissues (Orloff & Handler, 1962; Light *et al.*, 1989) had no effect. Similarly benzimidazole guanidine which can open epithelial sodium channels (Zeiske & Lindemann, 1974) had no stimulatory effect in sweat glands. Although not included in the results, a number of attempts were made to modify the responses to agonists by preincubation with agents shown elsewhere to alter transporting charac-

teristics. These included prolactin, oestrogens and progesterone (Robertson *et al.*, 1986; Zeitlin *et al.*, 1989). No changes were detected following these procedures.

Cyclic nucleotides and forskolin were without effect on SCC in sweat gland epithelia. Nevertheless an intact adenylate cyclase system was present which could be stimulated by forskolin (Table 3). Neither LBK nor isoprenaline caused an increase in cyclic AMP generation and there is evidence that isoprenaline produces an effect in salivary glands via a calcium signal (Cook *et al.*, 1988).

Considerable evidence was obtained to support the view that agonists promoting sodium absorption in the glands do so by mobilizing calcium. This includes (a) an increase in SCC following A23187 (including oscillations) and ionomycin, two calcium ionophores, (b) removal of agonist-induced currents by lanthanum ions, (c) presumed inhibition of phospholipase C with neomycin inhibits the response to LBK and (d) reversible inhibition of agonist-induced currents by EGTA. This latter implies, at least, that external calcium is required to maintain the response. More direct evidence comes from the measurement of $[\text{Ca}]_i$ in cell suspensions by fura-2 fluorescence. No major differences in the responses of normal and CF cells were found (Table 4). Although only few experiments were performed with some agonists there was but one exception to the rule that agonists increasing SCC also increased $[\text{Ca}]_i$. The exception was thapsigargin, an agent which apparently raises $[\text{Ca}]_i$ by inhibiting a calcium-ATPase which unbalances the pump-leak system in intracellular organelles (Thastrup *et al.*, 1990), allowing calcium to leak out thus raising cytosolic $[\text{Ca}]_i$. While this agent stimulates SCC in some epithelia (Brayden *et al.*, 1989) it has no effect on sweat gland epithelia (Table 1). Nevertheless $[\text{Ca}]_i$ is raised, apparently more so in controls than in CF cells. (Table 4). This result supports the notion that a temporally orchestrated and locationally correct increase in $[\text{Ca}]_i$ is required to trigger a physiological-type response (Putney, 1986). It remains a puzzle that A23187 can stimulate SCC and oscillations through a non-specific interaction, even though its effects are noticeably weaker than the receptor-operated increases.

Recently the presence of calcium-activated K-channels has been reported in sweat gland epithelia (Henderson *et al.*, 1990; Henderson & Cuthbert, 1990) which are considered to be located basolaterally. Opening of these K channels by increased $[\text{Ca}]_i$ effectively hyperpolarizes the apical membrane increasing the sodium influx. Similar mechanisms have been proposed before (Maruyama *et al.*, 1983; Mandel *et al.*, 1986) and it may well be that this is the major way in which sodium transport is enhanced by $[\text{Ca}]_i$. Indeed, in other situations membrane currents or potentials can be used to monitor intracellular $[\text{Ca}]_i$ with ion channels acting as an immediate reporter of $[\text{Ca}]_i$. For example, in smooth muscle cells, membrane potential and $[\text{Ca}]_i$ oscillate in unison (Yada *et al.*, 1986), while in pancreatic acinar cells, calcium-dependent chloride currents oscillate in phase with changes in $[\text{Ca}]_i$ (Opsichuk *et al.*, 1990). Thus there are strong precedents for our proposal. Alternatively, it is possible that apical sodium channels are directly regulated by $[\text{Ca}]_i$. However in another epithelium, the cortical collecting tubule, $[\text{Ca}]_i$ inhibits rather than promotes sodium entry (Palmer & Frindt, 1987). Barium ions proved to be a weak blocker of basal SCC and to be ineffective against maximally effective concentrations of LBK. With patch-clamping barium ions were found to have only a minor effect on K-channel open time and that only at depolarized potentials (Henderson & Cuthbert, 1990). It is not surprising, therefore, that in the relatively cruder experiments given here that effective block was not shown.

Finally it is necessary to consider if the receptors expressed *in vitro* are consistent with what is known about intact glands *in vivo*. Sweat gland coils receive both cholinergic and adrenergic innervation (Uno & Montagna, 1975; Sato, 1977; Uno, 1977), while vasoactive intestinal peptide (VIP) immunoreactive nerve endings appear to innervate both the coil and duct (Heinz-Erian *et al.*, 1985). Others have postulated that VIP

co-exists with acetylcholine in cholinergic nerves supplying the sweat gland (Hökfelt *et al.*, 1980). It is expected and found that the secretory coil cultures respond to both cholinergic and adrenoceptor agonists. However, while they do respond to carbachol and isoprenaline they have the characteristics of ductal cells, showing sodium absorption rather than a secretory process. On the other hand whole gland cultures were insensitive to VIP, as they were to other agents shown to increase cyclic AMP.

It is possible using primary cultures of whole glands that when the cells are disaggregated before seeding the culture wells there is a selection process (e.g. selective killing of one cell type) which results in epithelia with ductal characteristics. This possibility is negated by the experiments in which transporting epithelia were derived from either coils or ducts. Both tissues gave similar epithelia from a functional standpoint, and although the studies were limited there was no indication that the properties of epithelia were different from those derived from whole glands.

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