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Mechanisms of anion secretion in Calu-3 human airway epithelial cells by 7,8-benzoquinoline

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1 Cultured epithelial monolayers of Calu-3 human airway cells were used to measure anion secretion in response to a number of phenanthrolines and benzoquinolines, using short-circuit current measurements. Calu-3 cells are derived from serous cells of submucosal glands of the airways and are a target for conditions in which muco-ciliary clearance is compromised.

2 Compounds studied were 5,6-benzoquinoline, 5-chloro-1,10-phenanthroline, 7,8-benzoquinoline, 5-nitro-1,10-phenanthroline, benzo[c]cinnoline and 1,10-phenanthroline, which gave EC_{50} values of 34, 48, 123, 235, 192 and 217 μM, respectively. Of these, 7,8-benzoquinoline was chosen for further detailed study. Concentration–response relationships for all the compounds had Hill slopes greater than 1.

3 Permeabilisation of the apical surface of epithelia with nystatin in the presence of an apical to basolateral potassium ion gradient reduced the EC_{50} for 7,8-benzoquinoline to 31 μM and altered the Hill slope to close to 1.

4 Using apically permeabilised epithelia it was shown that 7,8-benzoquinoline activates an intermediate-conductance calcium-sensitive potassium channel (KCNN4) and a cAMP-sensitive potassium channel (KCNQ1/KCNE3) in the basolateral epithelial membranes.

5 7,8-Benzoquinoline was shown to increase chloride conductance of apical epithelial membranes, presumed to be by activation of the cystic fibrosis transmembrane conductance regulator.

6 7,8-Benzoquinoline had a minor effect on cAMP accumulation in Calu-3 cells, probably by inhibition of phosphodiesterase, which may contribute to its effect on CFTR- and cAMP-sensitive potassium channels.

7 The usefulness of these novel actions in promoting secretion in airway submucosal glands is discussed.

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Keywords: Airway submucosal glands; 7,8-benzoquinoline; calcium-sensitive potassium channels; cAMP-sensitive potassium channels; Calu-3 cells; cystic fibrosis; cystic fibrosis transmembrane conductance regulator (CFTR); chloride secretion; epithelia

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; ChTX, charybdotoxin; DPC, diphenylamine carboxylate; EBIO, 1-ethyl-2-benzimidazolinone; EMEM, Eagle's minimum essential medium; IBMX, isobutylmethylxanthine; KHS, Krebs Henseleit solution (also PGK, potassium gluconate Krebs and SGK, sodium gluconate Krebs); SCC, short-circuit current

Introduction

The expression of the cystic fibrosis transmembrane conductance regulator (CFTR) in the human lung is predominantly in the serous cells of the submucosal glands (Engelhardt *et al*., 1992). These cells elaborate a secretion, which contains antimicrobial enzymes and peptides, important in maintaining a sterile environment in the airways (Basbaum *et al*., 1990). Serosal cell secretion is important for maintaining adequate mucociliary clearance that is severely impaired in cystic fibrosis (CF) (Pilewski & Frizzell, 1999).

Shen *et al*. (1994) described a cell line, Calu-3, that has the properties of the serous cells and could be cultured on permeable supports to form an epithelium capable of vectorial transport of anions in response to agents increasing cAMP or Ca_i^{2+} (Moon *et al*., 1997). Under basal conditions, the secreted anion is mainly bicarbonate (Lee *et al*., 1998) and remains so when stimulated by forskolin (Devor *et al*., 1999). However agents such as 1-ethyl-2-benzimidazolinone (EBIO) activate basolateral K^+-channels and hyperpolarize the cells. This has the consequence of inhibiting the electrogenic $Na(HCO_3)_2$ cotransporter in the basolateral membranes, thus preventing bicarbonate secretion. Under these conditions, chloride crosses the basolateral membranes using the NaK2Cl cotransporter and the transport can be inhibited by bumetanide. As serous cells are targets for therapy in conditions where mucociliary clearance is impaired, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease, cultured Calu-3 epithelia are an appropriate test vehicle for preliminary studies. Recently, a number of phenanthrolines and benzoquinolines were screened

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for anion secretory activity using murine colonic epithelia (Cuthbert, 2003). We have examined the effects of some of these agents on a more relevant epithelium, namely epithelia of cultured Calu-3 cells. Additionally, one of these, 7,8-benzoquinoline, has been examined in detail to discover the mechanisms by which anion secretion is stimulated. It is shown that 7,8-benzoquinoline has multiple actions that contribute to the anion secretory activity.

Methods

Calu-3 cell culture

Calu-3 cells were obtained from the American Type Culture Collection and were grown on 75 cm^2 culture flasks containing Eagle's minimum essential medium (EMEM; Vitacell, ATCC, Virginia, U.S.A.) with 10% foetal calf serum (Gibco BRL), kanamycin 100 μM ml^{-1} and fungizone 1.25 mg ml^{-1}. Cells were incubated at 37°C in humidified air containing 5% CO_2. Cells from confluent monolayers were collected by trypsinisation and subcultured either on Snapwell polycarbonate membrane inserts (1 cm^2, 0.4 μM pore size) (Costar UK Ltd, Buckinghamshire, U.K.) or 96-well plates. Cultures were refed every 3–4 days and used between 17 and 24 days after subculture for the inserts or after 4 days for the cells on 96-well plates. All experimental procedures were made with cells of passages between 3 and 10.

Short-circuit current recording

The detachable rings of the Snapwell inserts, bearing the cultured monolayers, were inserted into CHM5 Ussing chambers with associated electrodes (WPI, Herts, U.K.) and voltage clamped at zero potential using a WPI Dual Voltage Clamp-1000 (WPI, Herts, U.K.). Both sides of the epithelium were bathed in 5 ml of Krebs Henseleit solution (KHS) that was continually circulated through the half chambers, maintained at 37°C and continuously bubbled with 95% O_2/5% CO_2. Short-circuit currents (SCCs) were recorded continuously using an ADInstruments PowerLab/8SP (NSW 2154, Australia) and displayed on a computer screen.

Modifications of the standard SCC procedure

To examine effects on the basolateral membranes of Calu-3 epithelia, the apical membrane was treated with nystatin (180–360 mg ml^{-1}) until the SCC increased to a new steady state. In these experiments, the apical bathing solution was changed to potassium gluconate Krebs (PGK) and the basolateral solution to sodium gluconate Krebs (SGK), thus imposing a K^+ gradient in the apical to basolateral direction. For examining effects of agents on the apical membranes, the method described by Cuthbert (2001) was used. A high potassium containing solution (PGK) is used to depolarise the basolateral membrane while the apical membrane remains bathed in KHS, thus imposing an apical to basolateral Cl^- gradient.

KHS had the following composition (mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11.1 glucose (pH 7.4). The modified PGK (NGK) solutions contained (mM): 120 potassium gluconate (sodium gluconate), 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 4 $CaCl_2$ and 10 glucose.

Drugs added to epithelial monolayers were added to either both sides, apically or basolaterally as follows: all the benzoquinolines and phenanthrolines and EBIO were added both sides, while amiloride, nystatin and diphenylamine carboxylate (DPC) were added apically and bumetanide, charybdotoxin (ChTX) and XE991 were added basolaterally.

Cyclic AMP accumulation by Calu-3 cell monolayers

Calu-3 cells were grown on 96-well plates until confluent, washed and treated for 10 min with 7,8-benzoquinoline, forskolin or EBIO at 37°C in the presence or absence of isobutylmethylxanthine (IBMX), 100 μM, after which the cells were lysed with dodecyl trimethyl ammonium bromide to stop cAMP generation and the supernatant assayed. Thus the method measures any cAMP lost from the cells together with intracellular cAMP. Six wells were used for each drug concentration examined. The lysate was assayed using an enzyme immunoassay system (Biotrak EIA system; Amersham Biosciences UK, Bucks, U.K.). Briefly, aliquots of the lysate were transferred to a titre plate precoated with rabbit anti-cAMP antibody. There was competition between cAMP in the lysate and a coupled cAMP-peroxidase for binding to the antibody. The colour from the peroxidase reaction when the substrate (3,3′,5,5′-tetramethylbenzidine-H_2O) was added provided the quantitative measure required for the construction of a standard curve and the estimation of the cAMP content of the lysate. Samples of washed, untreated wells of Calu-3 cells were lysed and used for protein determination.

Measurements of intracellular Ca^{2+} concentration

Intracellular Ca^{2-} was investigated using Fluo-4 as the reporter molecule. Calu-3 cells grown on 75 cm^2 flasks were incubated with versene (Gibco) to disrupt the tight junctions and, after agitation, form a cell suspension. The cells were resuspended in EMEM with no added serum. Calu-3 cells were exposed to Fluo-4AM, 5 μM in KHS, for 60 min followed by washing and resuspension in EMEM, all at 37°C. A period of 30 min was allowed for de-esterification. Emission fluorescence was measured at 516 nm with excitation at 488 nm each with a bandwidth of 5 nm. We avoided a ratiometric method using Fura-2, as phenanthroline-like compounds show strong absorption at wavelengths up to 350 nm. The fluorescence changes caused by 7,8-benzoquinoline have been compared to those for ionomycin. No attempt was made to convert the fluorescence measurements to actual Ca_I^{2+} values.

Materials

The following were obtained from Sigma-Aldrich Co. Ltd, Poole, Dorset, U.K. bumetanide, IBMX, nystatin, ChTX, and all the phenanthroline and benzoquinoline derivatives. Fluo-4AM was from Molecular Probes Europe BV, Leiden, Netherlands and forskolin was from Calbiochem. XE991 was a gift from Dr B.S. Brown, Dupont Pharmaceutical Company. EBIO (1-ethyl-2-benzimidazolone) was from Tocris, Bristol, U.K. and DPC from Lancaster Synthesis, Lancashire, U.K.

Statistics

A standard Student's *t*-test was used to compare mean values, a *P*-value of less than 0.05 being considered significant.

Results

General properties of Calu-3 monolayers

Bathed in KHS, bubbled with 95% O_2/5% CO_2 and maintained at 37°C Calu-3 monolayers had a basal SCC of $22.3 \pm 0.9\,\mu A\,cm^{-2}$ (range $3.2-51.8\,\mu A\,cm^{-2}$) ($n=100$) and a resistance of $327.7 \pm 15.7\,\Omega\,cm^2$ (range $59-1000\,\Omega\,cm^2$) ($n=100$). The resistances include the resistance of the permeable support that was less than $10\,\Omega\,cm^2$. The average age of the cultures was 21.6 ± 0.3 days (range 17–25 days) ($n=100$).

Quantitative concentration–response relationships of Calu-3 monolayers to benzoquinolines and phenanthrolines

Concentration–response curves to a number of phenanthrolines and benzoquinolines were obtained by cumulative addition of the agents to both the apical and basolateral solutions bathing Calu-3 monolayers. Two examples of actual SCC recordings are shown in Figure 1 for 5,6-benzoquinoline and 7,8-benzoquinoline. The peak at each concentration was used to construct the curves. For comparison, the inset in Figure 1a shows the response to a single concentration of 7,8-benzoquinoline. Note the responses to both agents are terminated by the addition of 20 μM bumetanide, to the basolateral side of the epithelium. While low concentrations of these agents produce a maintained increase in current, responses to higher concentrations are not maintained at their peak values, but eventually plateau at a steady state. The effects of 7,8-benzoquinoline were reversible by washing and could be repeated.

Between three and 10 individual Calu-3 monolayers were used to construct concentration–response curves for each compound examined. Mean EC_{50} values were obtained by simple interpolation of the individual curves. Figure 2 shows results for six different compounds and the EC_{50} values are given in Table 1. No attempt has been made to fit the curves to any model since, as later discussed, the compounds have more than one action. Several other features are revealed by the curves given in Figure 2. The slopes of the steepest sections of the curves have Hill slopes greater than 1. A second feature shown clearly by four of the compounds was inhibition of SCC at high concentration. Generally, concentration–response curves were made using one batch of Calu-3 monolayers, which may be responsible for some of the variation in the sizes of the maximal responses (see Discussion). To investigate the mechanism of action of this group of compounds, 7,8-benzoquinoline was chosen for detailed studies.

Potentiation of 7,8-benzoquinoline by forskolin

Agents that increase epithelial chloride secretion by activating basolateral K^+-channels are potentiated by other agents that activate apical chloride conductance (MacVinish *et al*., 1993).

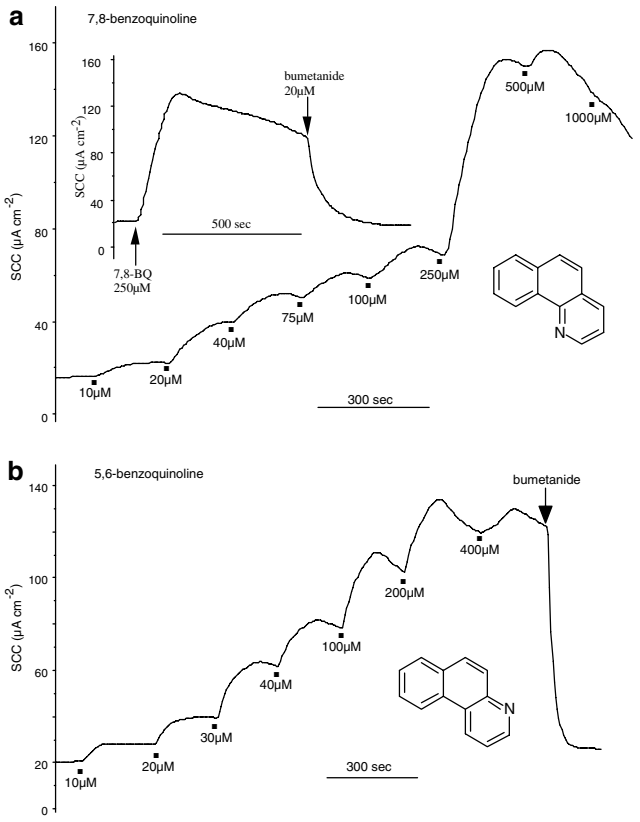

Figure 1 Concentration–respone relations in Calu-3 monolayers. SCC responses in Calu-3 monolayers following the cumulative addition of 7,8-benzoquinoline (a) and 5,6-benzoquinoline (b) added to both sides of the monolayers. (b) After the last concentration of 5,6-benzoquinoline had been added, 20 μM bumetanide was added to the basolateral solution. (a) Inset showing response of a Calu-3 monolayer to a single concentration of 7,8-benzoquinoline, 250 μM, the effects being terminated by bumetanide, 20 μM, applied basolaterally. Chemical structures of the two agents are shown.

For this reason, we investigated if activation of apical CFTR potentiated the effects of 7,8-benzoquinoline. 7,8-Benzoquinoline was used at two concentrations, 50 and 200 μM in the presence and absence of forskolin, 0.1 μM (Figure 3). Monolayer cultures were used in pairs, one of which was exposed to forskolin, 0.1 μM, and each was allowed to reach a steady state before 7,8-benzoquinoline was added. The SCC increase to forskolin in the two groups was not different being $21.2 \pm 3.1\,\mu A\,cm^{-2}$ ($n=5$ in (a)) and $21.7 \pm 3.4\,\mu A\,cm^{-2}$ ($n=5$ in (b)).

Three parameters were measured namely the peak height of the responses to 7,8-benzoquinoline, the plateau responses at a fixed time and finally the area under the SCC response, the latter corresponding to the total charge transfer during the response. All three parameters were potentiated by forskolin when the low (50 μM) concentration of 7,8-benzoquinoline was used but only the peak height was potentiated when the higher concentration (200 μM) was applied after forskolin. The failure to potentiate at high concentration may mean that the effect of forskolin on transport has already been activated by high concentrations of 7,8-benzoquinoline. Consequently, it was necessary to investigate separately the actions of the benzoquinoline on both the apical and basolateral faces of Calu-3 epithelia.

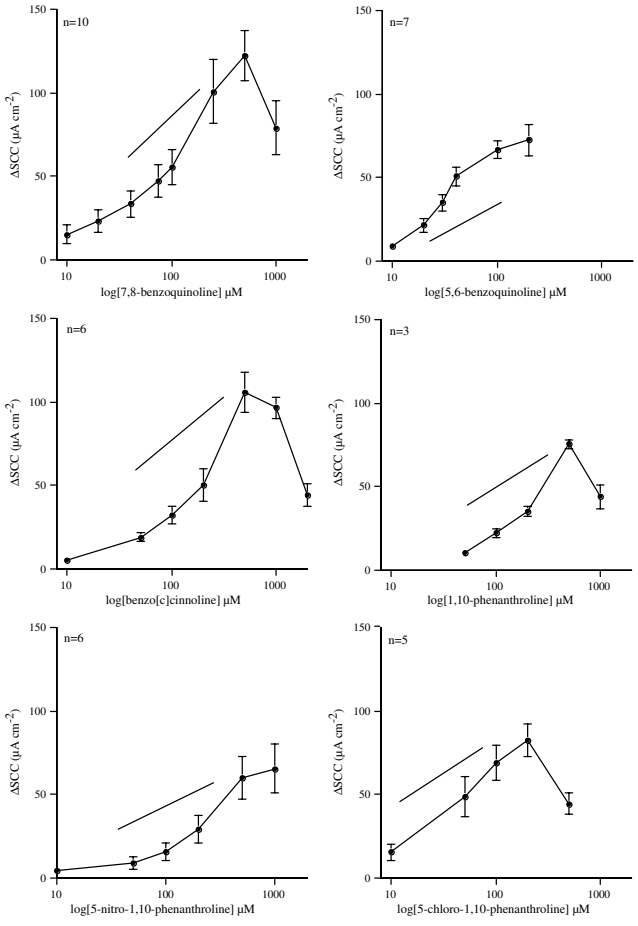

Figure 2 Concentration–respone curves for a number of phenanthroline and benzoquinoline analogues. Mean values ± s.e. are shown and the numbers of observations are indicated on each curve. The short straight lines simply indicate the slope of a concentration–response curve in the midrange with a Hill slope, $n_h = 1$. Note that in all instances, these slopes are less steep than the steepest parts of the actual data.

Table 1 EC_{50} values for phenanthrolines and benzoquinolines obtained on Calu-3 monolayers

Compound	*Calu-3 EC_{50}* (μM)
5,6-Benzoquinoline	33.9 ± 6.2 (7)
5-Chloro-1,10-phenanthroline	48.4 ± 8.7 (5)
7,8-Benzoquinoline	123.2 ± 14.0 (10)
5-Nitro-1,10-phenanthroline	235.2 ± 25.5 (6)
Benzo[c]cinnoline	192.3 ± 30.2 (6)
1,10-Phenanthroline	217.0 ± 26.0 (3)

Mean values ± s.e. are given with *n* values shown in parentheses.

Effects of 7,8-benzoquinoline on the basolateral membrane of Calu-3 monolayers

To examine the effects of 7,8-benzoquinoline on the basolateral membrane of Calu-3 monolayers, the apical membrane was permeabilised with nystatin in the presence of an apical to basolateral K^+ gradient as described in the Methods section. Application of nystatin caused a slow increase in SCC taking 30–40 min to reach a plateau value. Addition of 250 μM 7,8-benzoquinoline at this time caused a further rapid increase in SCC, the average value being 194.3 ± 18.6 μA cm^{-2} ($n = 4$). Addition of ChTX, 50 nM, reduced the SCC by 195.4 ± 13.7 μA cm^{-2} ($n = 4$) with a smaller additional effect to XE991, 30 μM, whether given before or after ChTX. The equivalence of the 7,8-benzoquinoline and ChTX effects suggests that 7,8-benzoquinoline activates a ChTX-sensitive K^+-channel in the basolateral membrane (Figure 4). However, this argument can only be sustained if it be first shown that the current increase to nystatin is unaffected by either XE991 or ChTX. This proved not to be so as both ChTX and XE991 had some effect on the nystatin current before 7,8-benzoquinoline was added. To quantify this, the blockers were added after nystatin alone. The mean response to nystatin (360 μg ml^{-1}) was 141.7 ± 31.7 μA cm^{-2} ($n = 10$). Addition of ChTX or XE991 caused, respectively, reductions of 57.2 ± 23.0 μA cm^{-2}

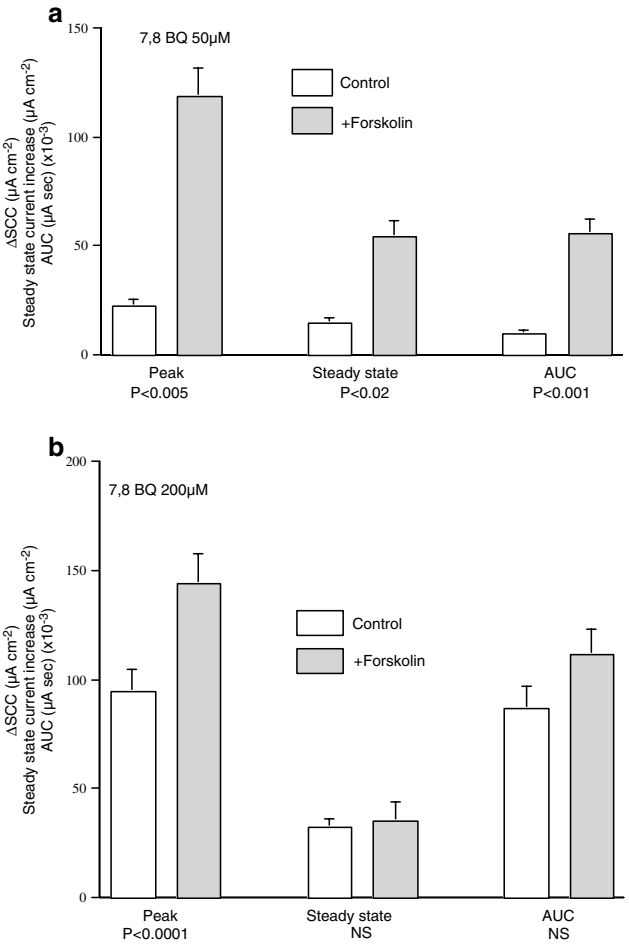

Figure 3 Effects of forskolin on the responses to 7,8-benzoquinoline. Calu-3 monolayers were exposed either to forskolin (0.1 μM) or used as controls. After a steady state had been reached, the monolayers were exposed to 7,8-benzoquinoline (50 μM (a) or 200 μM (b) and the SCC recorded for a further 30 min (a) or 20 min (b). The peak increases in SCC caused by 7,8-benzoquinoline (in μA cm^{-2}), the plateau increases in SCC at either 30 or 20 min (in μA cm^{-2}) and the area under curve (AUC) (in μA s) are shown. The value of 100 on the vertical scale corresponds to 1.04 μEq. Note all three parameters are potentiated at the lower concentration of 7,8-benzoquinoline, but only the peak SCC is potentiated at the upper concentration. The SCC increase caused by forskolin was not different in the two groups, being 21.2 ± 3.1 μA cm^{-2} ($n = 5$ in (a)) and 21.7 ± 3.4 μA cm^{-2} ($n = 5$ in (b)).

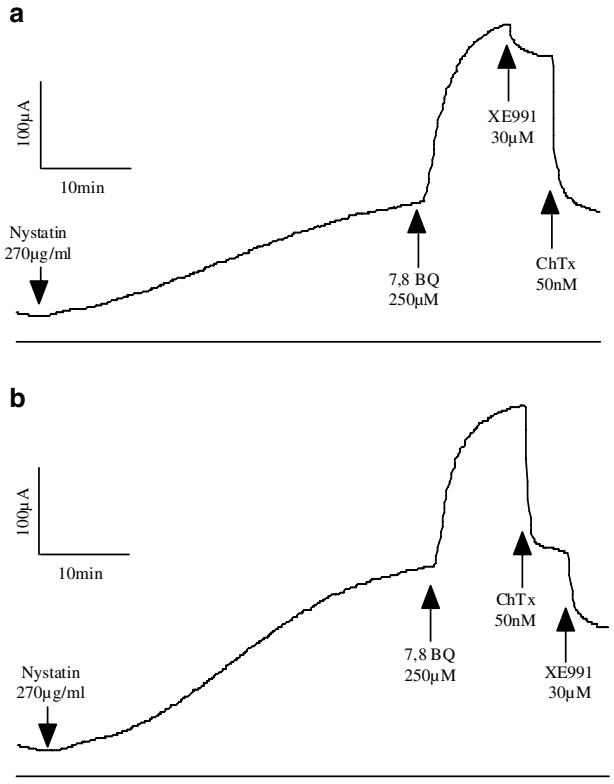

Figure 4 Effects of 7,8-benzoquinoline on basolateral membranes of Calu-3 cells. Responses of Calu-3 monolayers to 7,8-benzoquinoline, 250 μM, after apical treatment with nystatin, 270 μg ml^{-1}, in the presence of a K^+ gradient. After the response to 7,8-benzoquinoline had developed, XE991, 30 μM, followed by ChTX, 50 nM, was added to basolateral side (a) and in the reverse order (b).

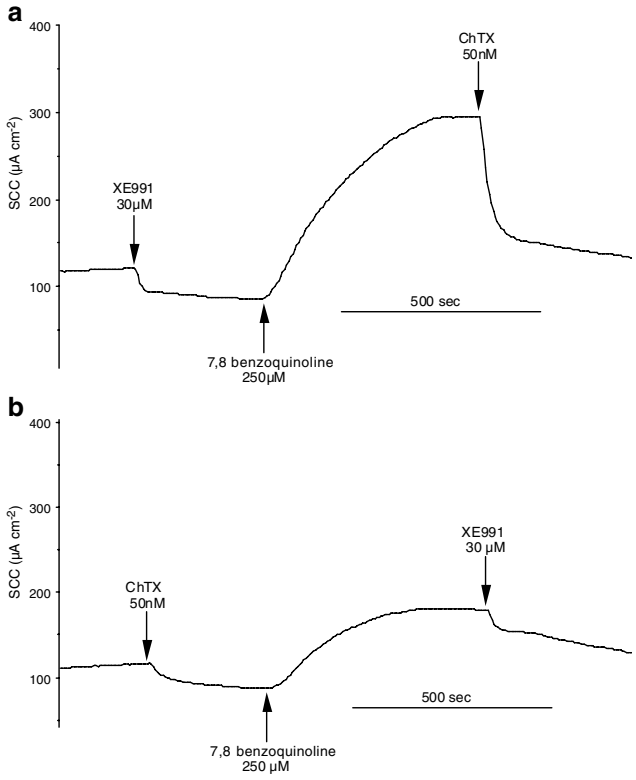

Figure 5 7,8-Benzoquinoline responses in nystatin-treated monolayers. Monolayers were treated with nystatin as before (not shown). (a) XE991, 30 μM, was added before 7,8-benzoquinoline, 250 μM, and ChTX, 50 nM, afterwards. (b) The order of addition of the blocking agents was reversed, that is, ChTX before 7,8-benzoquinoline and XE991 afterwards.

($n=5$) and $29.4\pm12.3\,\mu A\,cm^{-2}$ ($n=5$) showing that the K^+-channels in the basolateral membrane have some basal activity without stimulation. The total current removed by ChTX and XE991 combined is significantly less than the effects of ChTX added alone after 7,8-benzoquinoline (86.6 ± 26.1 ($n=5$) *versus* $195.4\pm13.7\,\mu A\,cm^{-2}$ ($n=4$), $P<0.012$) indicating that 7,8-benzoquinoline activates basolateral K^+-channels.

A further series of experiments illustrated in Figure 5 were carried out in which one inhibitor was added after nystatin but before 7,8-benzoquinoline, after which the second inhibitor was added. The propositions were, first, that any current generated by 7,8-benzoquinoline after nystatin plus XE991 was due to ChTX-sensitive K^+-channels. Similarly, any current generated by 7,8-benzoquinoline after nystatin and ChTX was due to XE991-sensitive K^+-channels. 7,8-Benzoquinoline gave an SCC increase of $162.5\pm14.5\,\mu A\,cm^{-2}$ ($n=5$) after nystatin and XE991. Subsequent addition of ChTX reduced the SCC by $167.9\pm22.8\,\mu A\,cm^{-2}$ ($n=5$), a value not significantly different from that due to benzoquinoline and substantially larger than the current reduction due to ChTX in the absence of 7,8-benzoquinoline (167.9 ± 22.8 ($n=5$) *versus* $57.2\pm23.0\,\mu A\,cm^{-2}$ ($n=5$), $P<0.01$). Similarly, when 7,8-benzoquinoline was given after nystatin and ChTX, the current increase was $137.4\pm65.8\,\mu A\,cm^{-2}$ ($n=5$), not significantly different from the reduction in current subsequent to the addition of XE991 of $149.2\pm85.6\,\mu A\,cm^{-2}$ ($n=5$). Therefore, it can be concluded that while addition of nystatin alone generates a current partially sensitive to both ChTX and XE991, 7,8-benzoquinoline further activates both ChTX- and XE991-sensitive K^+-channels in the basolateral membrane.

As the nystatin-treated epithelia are essentially 'basolateral membrane only' preparations, it was of interest to compare the concentration–response relationship to 7,8-benzoquinoline in this situation with that shown in Figure 2 for control epithelia. Five measurements were made of the concentration–response relationship in nystatin-treated membranes. In this series, the maximal response to 7,8-benzoquinoline, added cumulatively, was $79.5\pm17.4\,\mu A\,cm^{-2}$ ($n=5$). Each curve was analysed to provide an EC_{50} value and the mean value for five curves was $37.1\pm8.9\,\mu M$ ($n=5$), significantly different from the value for Calu-3 monolayers bathed in KHS (37.1 ± 8.9 *versus* $123.2\pm14\,\mu M$ ($n=10$) (Table 1), $P<0.002$). Furthermore, the slope of the concentration–response curve for nystatin-treated epithelia was less steep and close to unity. To compare the relationships for the two conditions, the responses were converted to percentages of the maximal response and plotted on the same scale (Figure 6). EC_{50} values from the composite curves for the two conditions were respectively 31 and 116 μM, close to the mean estimates given earlier.

Effects of 7,8-benzoquinoline on the apical membrane of Calu-3 monolayers

To examine the effects of 7,8-benzoquinoline on the apical membrane, the basolateral membrane was exposed to high

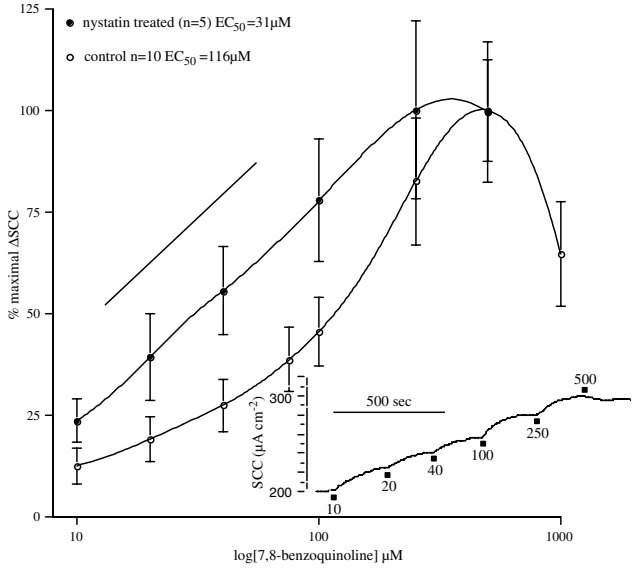

Figure 6 Concentration–respone curves to 7,8-benzoquinoline expressed as a percentage of the maximal response. The curves for Calu-3 monolayers bathed in KHS and for monolayers pretreated with nystatin, 270 μg ml^{-1}, and in the presence of an apical to basolateral K^+ gradient are shown. EC_{50} values from interpolation of the curves are 116 and 31 μM, respectively. The short solid line indicates the gradient for a Hill slope of 1. The inset shows the responses to 7,8-benzoquinoline (in μM) in one nystatin-treated epithelium. Mean values ± s.e. are shown.

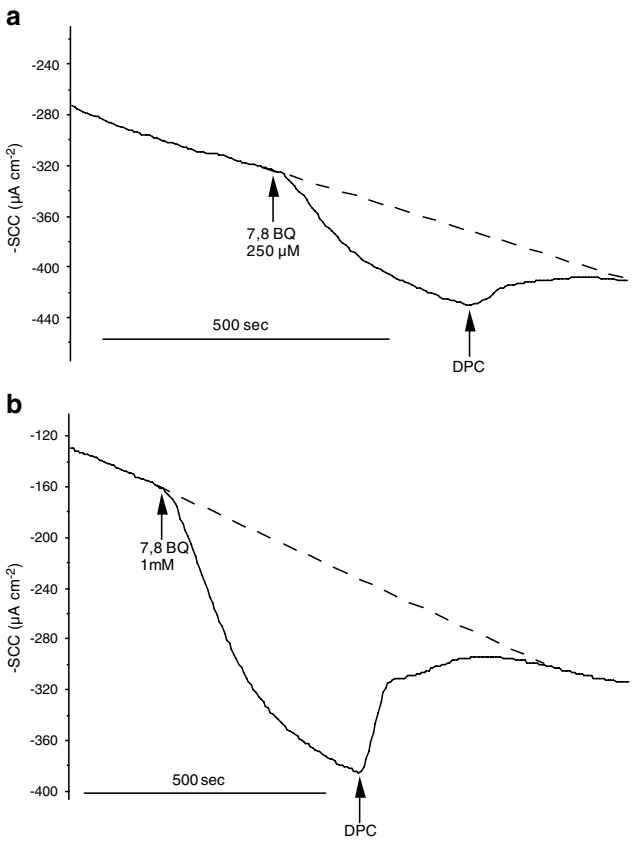

Figure 7 Responses to 7,8-benzoquinoline in potassium depolarised Calu-3 monolayers in the presence of an apical to basolateral chloride gradient. (a, b) Increases in −SCC in response to 7,8-benzoquinoline. Addition of DPC, 1 mM, apically reversed the responses.

potassium solution to create an 'apical membrane only' preparation (Cuthbert, 2001). In the presence of an apical to basolateral Cl^- gradient and under-short circuit conditions, the monolayers displayed an immediate negative current of −200–300 μA cm^{-2}, which showed a slow increase (Figure 7). Agents activating an apical chloride conductance are expected to increase the negative SCC. The average slope of the negative SCC (−SCC) increase before addition of 7,8-benzoquinoline, 250 μM, was -0.121 ± 0.016 μA s^{-1} ($n = 10$) that increased to -0.447 ± 0.089 μA s^{-1} ($n = 6$) ($P < 0.0004$) after addition. When 7,8-benzoquinoline, 1 mM, was used, the slope increased to 0.873 μA s^{-1} (mean of two) (Figure 7). To gain further evidence that the increase in slope was due to an increase in chloride conductance of the apical membrane, a number of controls were performed. First, addition of Ba^{2-} ions to the apical solution had no effect, suggesting that the conductance increase was not due to an increase in basolateral to apical potassium ion movement. Furthermore, addition of DPC, 1 mM, the nonspecific chloride channel blocker, to the apical side reversed the effect of 7,8-benzoquinoline to values expected, making allowance for the −SCC increase with time. Finally, other agents, such as EBIO and forskolin, known to increase apical membrane chloride conductance (Devor *et al*., 1996; Cuthbert *et al*., 1999) behaved similar to 7,8-benzoquinoline (data not shown). To further justify the claim that 7,8-benzoquinoline increased the chloride conductance of the apical membrane in Calu-3 monolayers, DPC, 1 mM, was added before 7,8-benzoquinoline. Two experiments, shown in Figure 8, are informative in this respect. In both, addition of DPC caused a rapid decrease in −SCC, after which the slow increase in −SCC resumed but at a slower rate. This result argues that some, but not all, of the −SCC is due to chloride ions moving in an apical to basolateral direction. Addition of 7,8-benzoquinoline in the presence of DPC either produced no increase in −SCC (Figure 8a) or only a small response (Figure 8c). After washing away both DPC and 7,8-benzoquinoline, the same monolayers were able to respond to 7,8-benzoquinoline with an increase in −SCC that was reversed by DPC (Figure 8b,c). The conclusion is that 7,8-benzoquinoline affects an apical chloride conductance as well as both XE991- and ChTX-sensitive potassium conductances in the basolateral membrane.

Effects of 7,8-benzoquinoline on cAMP content in Calu-3 monolayers

Calu-3 cells were seeded onto 96-well titre plates and used to measure total cAMP after stimulation with 7,8-benzoquinoline as described in the Methods section. Forskolin and EBIO were used as controls. The values of total cAMP per well were derived by interpolation on a standard curve and represent not only the cAMP present within the cells but also that released into the medium. The data are given in Figure 9 and show the results from two conditions, that is, either without or with the inclusion of IBMX, 100 μM, in the medium to inhibit phosphodiesterase. As expected, the values in the presence of IBMX are elevated over those in its absence.

In the absence of IBMX (Figure 9a), no increase in total cAMP was detectable with either 0.1 μM forskolin or with 100 μM 7,8-benzoquinoline. However, when the concentration

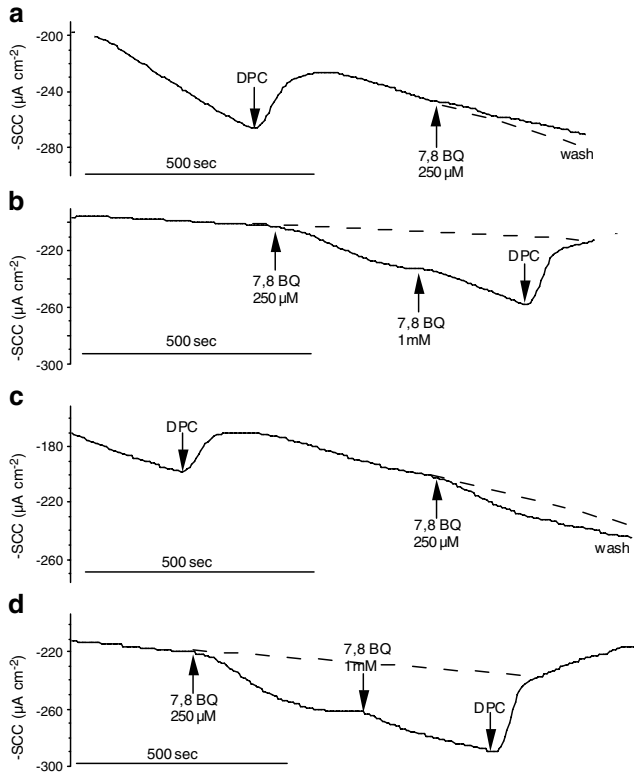

Figure 8 Further responses made with potassium depolarised Calu-3 monolayers in the presence of an apical to basolateral chloride gradient. Two separate monolayers were used to show that DPC prevented responses to 7,8-benzoquinoline (a, c) after which they were washed and re-exposed to 7,8-benzoquinloine (b, d). In (a) DPC, 1 mM, added apically prevented any further increase in −SCC to 7,8-benzoquinoline, 250 μM, while in (c) there was a small response. After extensive washing, the responses to 7,8-benzoquinoline were restored (b, d) and their responses reversed by addition of DPC.

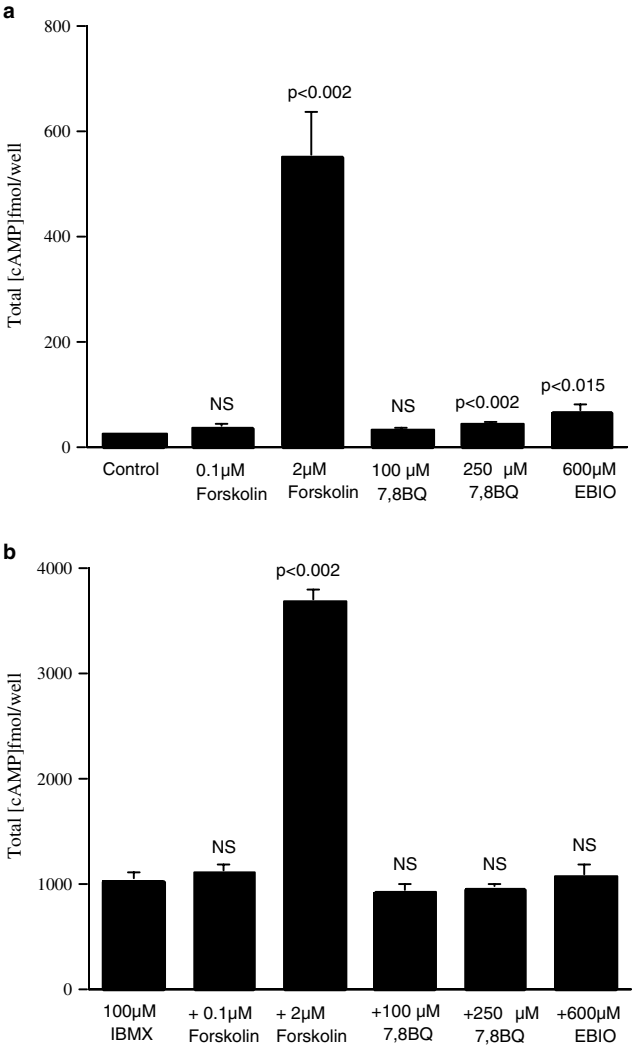

Figure 9 Cyclic AMP content of Calu-3 cells. Values for the total cAMP (cells and supernatant) in Calu-3 cells (as pmol/well) measured in the absence (a) and presence (b) of IBMX, 100 μM, are shown. Cells were stimulated with forskolin (0.1 and 2.0 μM), 7,8-benzoquinoline (100 and 250 μM) and EBIO (600 μM). Mean values $\pm$s.e. for $n=6$ are shown. The protein content per well was 30.8 ± 1.2 μg.

of 7,8-benzoquinoline was raised to 250 μM, there was a small, but significant increase, from 19.7 ± 2.4 pmol well^{-1} in the control to 42.9 ± 3.9 pmol well^{-1} with the benzoquinoline present. EBIO, 600 μM, produced an even greater increase compared to control raising the concentration to 66.2 ± 12.3 pmol well^{-1}. These increases were marginal compared to the increase produced by 2 μM forskolin that gave 550.4 ± 83.0 pmol well^{-1}. In the presence of IBMX (Figure 9b), the values for all conditions were raised. For example, in the presence of IBMX alone, the cAMP content per well was over 50 times larger than in its absence, suggesting that cAMP is continually produced in Calu-3 cells without other stimulation. Only 2 μM forskolin showed any significant increase above control values in the presence of IBMX, while 7,8-benzoquinoline and EBIO showed no increase over IBMX alone.

Effect of 7,8-benzoquinoline on Ca_I^{2+} in Calu-3 monolayers

No increase in fluorescence was recorded with 7,8-benzoquinoline in five separate experiments with suspensions of Calu-3 containing Fluo-4. Indeed most suspensions showed a minor decrease in fluorescence on addition of the benzoquinoline, whereas ionomycin was able to increase the fluorescence. Fluorescence measurements were made after 200 s of control observation or drug exposure. The basal fluorescence was 5.49 ± 0.59 ($n=5$), whereas after 7,8-benzoquinoline it was 5.13 ± 0.51 ($n=5$, $P<0.03$, paired t-test), but increased to 6.2 ± 0.67 ($n=5$, $P<0.008$ compared to basal value, paired t-test) after ionomycin. An example of Fluo-4 fluorescence changes in Calu-3 cells in response to 7,8-benzoquinoline and ionomycin is given in Figure 10.

Discussion

Our major aim in this work was to examine the mechanism by which 7,8-Benzoquinoline increases SCC in Calu-3 monolayers. 7,8-Benzoquinoline is a member of a series of phenanthrolines and benzoquinolines that were initially screened for their effect on anion secretion on murine colonic epithelia (Duszyk *et al.*, 2001; Cuthbert, 2003). All have been shown to enhance chloride secretion in the epithelium of the

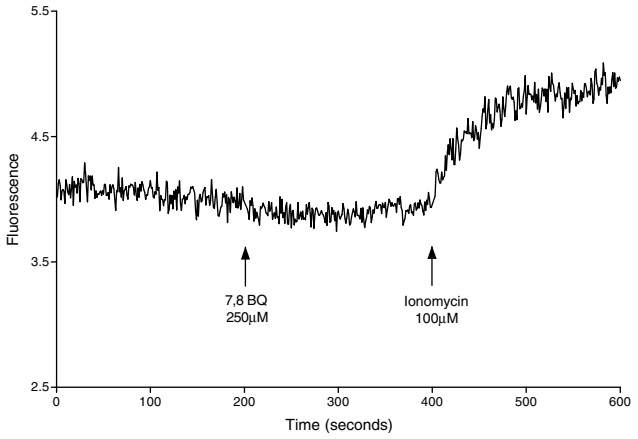

Figure 10 Measurements of intracellular Ca^{2+} in Calu-3 cells. The fluorescence due to Fluo-4 at 516 nm, with excitation at 488 nm, of a Calu-3 cell suspension is shown. 7,8-Benzoquinoline, 250 μM, and ionomycin, 100 μM, were added as indicated.

mouse colon and it has been argued that compounds like these may be useful in clinical conditions such as cystic fibrosis and chronic obstructive pulmonary disease (Singh *et al*., 2001). For this reason, these agents have been re-examined for activity on a more relevant epithelium, namely Calu-3 cell epithelial monolayers. Calu-3 cells are derived from a human lung adenocarcinoma and are considered to have the properties of the serous cells found in submucosal glands of the airways, and contain the richest source of CFTR in the pulmonary system (Shen *et al*., 1994). All the six compounds investigated in this study produced substantial increases in SCC, sensitive to the addition of bumetanide to the basolateral side of the epithelium, indicating that the active transport of chloride is responsible. Fluid secretion dependent upon the electrogenic transport of anions is considered a major responsibility of serous cells *in situ* as this creates fluid necessary for maintaining muco-ciliary clearance in the airways, a function greatly depressed in CF, and directly linked to the absence of CFTR in this condition (Pilewski & Frizzell, 1999).

It is thought that submucosal glands normally secrete a bicarbonate-rich fluid with a slightly alkaline pH (Lee *et al*., 1998), which later becomes slightly acidic by modification in the proximal part of the glands or by the surface epithelium (Inglis *et al*., 2003). Devor *et al*. (1999) have discussed the switch from bicarbonate to chloride secretion when Calu-3 monolayers are stimulated with EBIO.

Concentration–response curves were determined by cumulative addition of compounds. No attempt was made to fit the concentration–response curves to a simple drug–receptor model since, as is shown, the compounds have at least two separate actions. The concordance between the estimates of EC_{50} values for Calu-3 cells and mouse colon epithelium was close in some instances (5,6-benzoquinoline, 7,8-benzoquinoline, 5-nito-1,10-phenanthroline and benzo[c]cinnoline) and discrepant in others (5-chloro-1,10-phenanthroline and 1,10-phenanthroline) (see Cuthbert, 2003). 5,6-Benzoquinoline was the most active in both tissues and is roughly equipotent to dichloro-EBIO ($EC_{50} = 45\ \mu$M), the most potent derivative so far developed from EBIO, estimated from SCC measurements on T84 monolayers (Singh *et al*., 2001). The steepest parts of the concentration–response curves had Hill slopes greater than 1, and in some instances, as with 1,10-phenanthroline and 5,6-benzoquinoline, the deviation was pronounced. In the mouse colon 1,10-phenanthroline had a Hill slope of 4.9 (Duszyk *et al*., 2001). Steep Hill slopes are generally a sign of a cooperative interaction between drug and receptor, but here it is more likely that it is the result of a synergistic interaction between effects at the apical and basolateral membranes (see below). Figure 2 demonstrates that the maximal responses achieved by the different compounds are unequal. In another study with cultured human bronchial epithelial cells (Caci *et al*., 2003), the maximal SCC responses were dependent on the basal SCC and were attributed to different basal adenylate cyclase activities. Here when the basal current was doubled with a low concentration of forskolin, responses to 7,8-benzoquinoline were potentiated. Thus the differences in maximal responses may be more related to basal cAMP levels than to differences in activity.

Preliminary data showed that not only was bumetanide able to inhibit the responses to 7,8- and 5,6-benzoquinoline but also that the responses were sensitive to agents blocking basolateral epithelial K^+-channels, such as ChTX and XE991. Thus not only is the NaK2Cl cotransporter necessary for transporting Cl^- in through the basolateral surface of the epithelium, but hyperpolarisation of the cells is important for maintaining a suitable electrochemical gradient for exit of Cl^- across the apical face. Whether the apical chloride conductance is normally sufficient to maintain a high level of chloride secretion without additional recruitment of chloride channels is unclear, although it has been argued that CFTR is substantially activated under baseline conditions (Shen *et al*., 1994). Therefore, we have investigated whether 7,8-benzoquinoline has separate actions on the apical and basolateral surfaces of Calu-3 monolayers.

Using nystatin to permeabilise the apical membrane, making it freely permeable to small ions, and in the presence of an apical to basolateral K^+ gradient, the effect of agents on basolateral K^+ conductance can be assessed. It was shown that two blockers of epithelial K^+-channels, namely ChTX and XE991, caused partial inhibition of the K^+ current under resting conditions. ChTX blocks the intermediate-conductance Ca^{2+}-sensitive K^+-channel, KCNN4, while XE991 ($EC_{50} = 20\ \mu$M, MacVinish *et al*., 2001) blocks the cAMP-sensitive K^+-channel, KCNQ1/KCNE3. 7,8-Benzoquinoline caused an increase in K^+ current that was larger than the total current removed by ChTX and XE991 combined in the absence of benzoquinoline and, furthermore, this current was sensitive to ChTX. By adding either ChTX or XE991 before 7,8-benzoquinoline, but after nystatin, it was shown that 7,8-benzoquinoline activated both ChTX- and XE991-sensitive K^+-channels. While ChTX is not entirely specific for KCNN4, others have shown the presence of ChTX-sensitive intermediate-conductance Ca^{2+}-sensitive K^+-channels in Calu-3 cells variously described as hIK1, hSK4 and hIK using earlier nomenclatures (Ishii *et al*., 1997; Joiner *et al*., 1997; Jensen *et al*., 1998; Catterall *et al*., 2002). The presence of cAMP-sensitive K^+-channels in these cells is highly likely as mRNA for the channel, KCNQ1 and its subunit, KCNE3 have been demonstrated (Cowley & Linsdell, 2002). Our results differ in an important way from those of Devor *et al*. (1999), who reported that EBIO affected only KCNN4 channels in the basolateral membranes of Calu-3 epithelia. However, in another report from the same group (Singh *et al*., 2001), 293B, another cAMP-sensitive K^+-channel inhibitor, was

reported to cause a small effect on currents generated by dichloro-EBIO.

Turning to the apical membrane, we used the potassium-depolarised epithelium to examine for effects of 7,8-benzoquinoline. In this configuration, the basolateral membrane is electrically silent and the voltage clamp is applied across the apical membrane in the presence of an apical to basolateral Cl^-gradient (Cuthbert, 2001). Since CFTR is thought to be the only Cl^- conductance in the apical membrane of Calu-3 cells (Wine *et al*., 1994), agents increasing the Cl^- conductance presumably act through this mechanism. An increase in Cl^- conductance is indicated by an increase in negative SCC. Addition of a nonspecific blocker of Cl^--channels, DPC, partially reversed the −SCC indicating that some Cl^--channels were patent without prior stimulation. 7,8-Benzoquinoline, as well as other agents EBIO and cAMP (given as forskolin) produced increases in −SCC (data not shown), indicating activation of apical chloride channels, presumably CFTR.

The mechanisms by which 7,8-benzoquinoline activates basolateral K^+-channels and apical Cl^--channels can be either direct or indirect. We showed that both 7,8-phenanthroline and EBIO caused the accumulation of cAMP in Calu-3 monolayers. Whether this was by stimulation of adenylate cyclase or by inhibition of phosphodiesterase is uncertain; however, in the presence of IBMX to inhibit phosphodiesterase, no further accumulation above control values was apparent. This result favours the suggestion that phosphodiesterase inhibition is the mechanism by which 7,8-benzoquinoline and EBIO increase cAMP production. The 50-fold increase in cAMP accumulation in the presence of IBMX indicates that adenylate cyclase is turning over in the absence of any other stimulation. This fits well with the findings that the basal current in Calu-3 monolayers is electrogenic HCO_3^-secretion (Lee *et al*., 1998) that can be further activated by forskolin or cAMP. The question is whether the effect of 7,8-benzoquinoline on cAMP accumulation is sufficient to affect either CFTR or basolateral cAMP-sensitive K^+-channels. Since 7,8-benzoquinoline, 100 μM, failed to produce any significant increase in cAMP accumulation, yet increased SCC by at least 50 $\mu A\,cm^{-2}$, it might be argued that it has actions independent of cAMP. However, we failed to see any cAMP accumulation to forskolin at 0.1 μM, yet it increased current by over 20 $\mu A\,cm^{-2}$ and potentiated the responses to low concentrations of 7,8-benzoquinoline (Figure 3). It is known that adenylate cyclase, protein kinase A and CFTR occur together in apical membrane microdomains in epithelial cells, thus allowing CFTR activation without an overall increase in cellular cAMP (Huang *et al*., 2001). Alternatively, low concentrations of 7,8-benzoquinoline may act exclusively on basolateral K^+-channels, explaining the potentiation by low forskolin concentrations. At higher concentrations of 7,8-benzoquinoline, causing cAMP accumulation, the potentiation by forskolin disappears. To determine whether 7,8-benzoquinoline has direct actions on either CFTR- or cAMP-sensitive K^+-channels will require a biophysical study of isolated membrane patches. We found no evidence to suggest that 7,8-benzoquinoline activated basolateral calcium-sensitive K^+-channels by raising Ca_i^{2+}, although this does not mean that its actions on this channel are calcium independent.

In tissues treated with nystatin on the apical side, in the presence of an apical to basolateral K^+ gradient, the concentration–response curve to 7,8-benzoquinoline was shifted to the left by a factor of 4. In this situation, the EC_{50} presumably more accurately represents the interaction of 7,8-benzoquinoline with basolateral K^+-channels. The slope of the concentration–response curve was close to a theoretical Hill slope of 1. This result is reminiscent of the increase in potency of dichloro-EBIO when examined by patch clamping compared to its effect on SCC, the latter depending on both apical and basolateral effects (Singh *et al*., 2001).

The usefulness of compounds that activate epithelial basolateral K^+-channels is their ability to promote anion secretion and hence generate the fluid for submucosal gland secretion. In the absence of any conductance pathway for anion exit, as in CF, K^+-channel activation alone will not achieve this. The most common mutation in CF is ΔF508 due to a missing triplet codon. The gene product ΔF508 CFTR fails to be trafficked to the cell membrane, but acts as surrogate CFTR if it can be made to do so. Agents that increase trafficking, minimally effective gene therapy or CF patients showing partial trafficking of the mutant protein (Kalin *et al*., 1999) are conditions in which effectiveness might be boosted with K^+-channel activators. However, when potassium channel activation increases anion secretion, hyperpolarisation also promotes electrogenic sodium absorption (Gao *et al*., 2001) and hence is profluid absorption. While this is so for the surface airway epithelium, it cannot apply to the Calu-3 monolayers since no pathway for electrogenic sodium absorption exists, although the evidence for submucous glands themselves remains open (Phillips *et al*., 2002).

Finally, benzo[c]quinolizinium (MPB) compounds have been described that increase trafficking of ΔF508 CFTR (Dormer *et al*., 2001). The basic chemical nucleus of these compounds is not dissimilar to the benzoquinolines described here and the possibility exists of a single molecule that activates trafficking as well as the relevant processes at both the apical and basolateral surfaces.

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